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L'INTROGRESSION DE L'ADN MITOCHONDRIAL CHEZ L'OMBLE DE FONTAINE, *Salvelinus fontinalis* DANS L'EST DU QUÉBEC: CARACTERISATION, DISTRIBUTION GÉOGRAPHIQUE ET SIGNIFICATION ÉVOLUTIVE

MITOCHONDRIAL DNA INTROGRESSION IN BROOK CHAR, *Salvelinus fontinalis* FROM EASTERN QUEBEC: CHARACTERIZATION, GEOGRAPHICAL DISTRIBUTION AND EVOLUTIONARY SIGNIFICANCE

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ABSTRACT

A population of brook char (*Salvelinus fontinalis*) from lac Alain situated in the Portneuf river system of eastern Quebec was discovered to be introgressed with the mitochondrial DNA (mtDNA) of the more northerly occurring Arctic char (*S. alpinus*). Individuals from this population resembled typical brook char based on allozyme analysis and several morphological and meristic characteristics. Based on RFLP analysis, all individuals were fixed for the Arctic char mtDNA haplotype, despite the present absence of Arctic char in the watershed. The geographical extent of mtDNA introgression was determined by the genetic assessment of populations from both within the Portneuf river system, as well as from neighboring watersheds. Introgressed populations were restricted to lakes occurring along one branch of the Portneuf river system, otherwise, elsewhere in the watershed, populations were that of genetically pure brook char. All populations tested from neighboring drainages were also genetically pure brook char. It is probable that a combination of biogeographical conditions coupled with positive selection for mtDNA introgression led to the present-day distribution of introgressed *S. fontinalis*.

The evolutionary importance of mtDNA introgression was investigated by using an experimental approach which involved the assessment of physiological performance of both introgressed and non-introgressed brook char at different levels of biological organization. This approach allowed any physiological advantage imparted by the mtDNA of an arctic species (via the proteins it encodes), to be identified in introgressed brook char. As a first step of this approach, the catalytic efficiency, as well as the thermal sensitivity of cytochrome C oxidase (CCO), an enzyme partly encoded by mtDNA was determined in red muscle mitochondria. Differential responses in catalytic efficiency and Q_{10} values between char groups were observed for CCO and suggest an influence by the mitochondrial genome on enzyme structure and function. Based on these enzymatic differences, the prediction at the sub-cellular level of mitochondrial function, was that introgressed *S. fontinalis* would have mitochondria which function better at cold temperatures. Indeed, introgressed *S. fontinalis* were found to have heightened mitochondrial capacity at low temperatures (6 °C) based on

the oxidation of malate and succinate in isolated red muscle mitochondria. These results provide supporting evidence for a mitochondrial genome influence on enzyme structure and function, as well as an influence at the sub-cellular level of mitochondrial function which is not neutral.

The aerobic capacity and swimming performance of fish were evaluated using energetic parameters determined by swimming respirometry. At low temperatures, introgressed *S. fontinalis* were found to have a metabolic scope for aerobic activity similar to that of non-introgressed *S. fontinalis*. Further analysis of the metabolic scope revealed the potential for accommodating feeding metabolism was also similar between fish groups. These findings suggest that there is no apparent physiological basis related to swimming metabolism that could be of selective value to introgressed *S. fontinalis*. Still, further studies should be undertaken that evaluate the possibility of such a basis existing at earlier stages in the life history of introgressed *S. fontinalis*.

Taken together the results from this study demonstrate, for the first time, that mtDNA variation resulting from introgressive events can be functionally important at the molecular and sub-cellular level and may be selectively maintained in natural populations. These findings are of particular interest to population and evolutionary biologists as mtDNA variation is traditionally believed to be of neutral consequence at all levels of biological organization.

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TABLE OF CONTENTS

List of Tables and Figures

RÉSUMÉ EN FRANÇAIS

Table des matières

I. Problématique

L'hybridation naturelle entre des espèces phylogénétiquement proches et occupant des niches thermiques différentes peut survenir à des endroits où leurs distributions se chevauchent. L'hybridation naturelle chez les poissons, plus particulièrement chez les poissons d'eau douce, se retrouve a une fréquence relativement grande comparativement aux autres groupes de vertébrés (Campton, 1987). La capacité des poissons à atteindre des niveaux plutôt élevés d'hybridation interspécifique est principalement due à la fertilisation externe. Cependant d'autres facteurs contribuent à la capacité d'hybridation élevée des poissons. Ceux-ci incluent des nombres inégaux d'espèces parentales dans des populations sympatriques de poissons, c'est-à-dire l'absence de partenaire sexuel appartenant à la même espèce et la compétiton pour les sites de fraie. Aussi, le contact secondaire accrû par

l'intervention humaine, particulièrement par des introductions accidentels de poissons, et le jeune âge des habitats d'eau douce formés depuis la dernière glaciation contribuent à la capacité d'hybridation élevée des poissons. (Campton, 1987; Billington and Hebert, 1991).

Une des rares conséquences de l'hybridation naturelle est l'introgression. L'introgression signifie que des gènes ou du matériel génétique nouveau d'une espèce sont transférés à une autre espèce (Verspoor and Hammar, 1991). Aussi longtemps que la progéniture hybride est fertile, le croisement de l'hybride avec un individu de l'une ou l'autre des populations parentales peut conduire à un transfert de gènes entre les taxa. L'hybridation introgressive peut donc donner lieu à des changements temporaires ou permanents dans le bagage génétique des populations qui s'hybrident, dépendemment du degré d'isolement reproducteur et du caractère assorti de l'accouplement. Bien qu'un sentier potentiel important pour le flux génique entre les espèces, le phénomène d'introgression chez les poissons est relativement rare et son impact sur la variation génétique est encore peu compris. Par conséquent, on en connaît que très peu sur la signification évolutive de l'hybridation introgressive. Il pourrait y avoir des avantages selectifs à posséder des gènes d'une autre espèce. De plus, ces avantages sélectifs pourraient contribuer au succès reproducteur de l'individu introgressé.

Dans le passé, des hypothèses sélectionnistes de ce type ont été proposées, mais n'ont jamais été testées spécifiquement. Il a été suggéré que le transfert interspécifique de gènes, comme il se produit lors de l'introgression, est un phénomène neutre et cette vision a généralement dominée la littérature (Dowling and Hoeh, 1991). L'acceptation de la neutralité sans considérer davantage l'adaptabilité a freiné notre compréhension des rôles relatifs des

processus déterministes et stochastiques comme forces dirigeantes du flux génique entre différents taxa (Arnold, 1992). Afin de tester rigoureusement l'hypothèse sélectionniste holistique, une approche expérimentale incluant des mesures de performance à différents niveaux d'organisation biologique est requise (Powers et al., 1991). Une telle approche a été développée et utilisée dans le passé afin de trouver les rôles adaptatifs potentiels de la variation génétique nucléaire des loci codant pour des enzymes. Cette approche pourrait, en théorie, être utilisée pour étudier la signification évolutive de la variation retrouvée au niveau de l'ADNmt.

La présente étude servira à développer une meilleure compréhension de la signification évolutive de l'hybridation introgressive chez les poissons. Elle examinera l'influence de gènes mitochondriaux intogressés sur la performance physiologique à différents niveaux d'organisation biologique. La démonstration d'une performance "supérieure" dans les populations introgressées aux niveaux moléculaire et cellulaire fournira une forte évidence que l'hybridation introgressive du génome mitochondrial est de nature non-neutre.

II. Hypothèse centrale

L'hypothèse centrale de mon travail est que les populations d'ombles de fontaine (*Salvelinus fontinalis*) introgressés possédant le génome mitochondrial de l'omble chevalier (*S. Alpinus*), une espèce à distribution plus nordique, bénéficient d'un avantage physiologique à basses températures comparativement aux populations d'ombles de fontaine génétiquement purs.

III. Objectifs

L'objectif principal de cette étude sera de tester l'influence génétique du génome mitochondrial sur les performances physiologiques des populations de *Salvelinus fontinalis* à basses températures afin de clarifier l'importance évolutive de l'introgression de l'ADNmt. Une approche comparative sera utilisée, impliquant l'identification des différences génétiques et physiologiques entre les *S. fontinalis* introgressés et les *S. fontinalis* non introgressés.

Les différences dans les génomes mitochondriaux peuvent se refléter au niveau moléculaire par des modifications dans la structure tertiaire des sous-unités des enzymes mitochondriales respiratoires. Ces dernières sont codées partiellement par le génome mitochondrial. Ces enzymes mitochondriales peuvent manifester des efficacités catalytiques différentes dues à des variations dans leur flexibilité. Notons que la flexibilité est transmise par la structure même des sous-unités enzymatiques. Au niveau subcellulaire, l'efficacité fonctionnelle des mitochondries peut être affectée puisque que la respiration cellulaire est dépendante de la respiration mitochondriale et donc des enzymes mitochondriales. Finalement, l'influence du génome mitochondrial sur la structure des sous-unités protéiques peut se manifester sur la capacité de performance de l'organisme.

Les objectifs spécifiques de cette étude incluront:

1. *Caractérisation morphologique et génétique des populations d'ombles de fontaine (S. fontinalis) introgressés.*

La population introgressée de *S. fontinalis* du lac Alain sera comparée à des populations pures d'ombles de fontaine et d'ombles chevaliers. La caractérisation incluera

des mesures morphologiques et méristiques, l'analyse du polymorphisme des longueurs de fragments d'enzymes de restriction (PLFR) sur l'ADN mitochondrial ainsi que l'analyse d'allozymes (ADN nucléaire). Ces résultats nous permettront de quantifier le degré d'introgression.

2. *Détermination de la distribution géographique des populations de S. fontinalis introgressés.* L'étendue géographique de l'introgression des populations de *S. fontinalis* sera déterminée dans la région de l'est du Québec où la population originelle a été pour la première fois découverte. Les populations originant du même bassin versant que la population du lac Alain ainsi que celles des bassins versants avoisinants seront évaluées en ce qui concerne l'introgression par une analyse de leurs génomes nucléaire et mitochondrial.

3. *Évaluation de la performance physiologique aux niveaux moléculaire et subcellulaire chez des individus S. fontinalis introgressés et non introgressés.*

La flexibilité structurelle d'une enzyme mitochondriale, la cytochrome C oxydase, sera déterminée en examinant les effets de changements de température sur l'activité enzymatique. L'influence de la structure de l'enzyme sur la fonction mitochondriale sera testée en suivant la consommation d'oxygène des mitochondries isolées de muscle rouge en présence de différents substrats métaboliques.

4. *Détermination de la performance de nage soutenue chez des populations de S. fontinalis introgressés et typiques acclimatés à basse température.*

La capacité aérobie au niveau individuel sera déterminée à l'aide de paramètres énergétiques. Ces dermiers seront onbtenus par des tests natatoires. Le registre métabolique

pour l'activité aérobie et la performance de nage seront comparés entre des individus *S. fontinalis* introgressés et non introgressés.

Globalement, nous prévoyons que les populations introgressées auront un potentiel physiologique supérieur à basses températures du aux avantages procurés par le génome mitochondrial d'une espèce plus nordique. La démonstration d'une performance physiologique supérieure chez la population introgressée de *S. fontinalis*, une performance susceptible d'être corrélée positivement au succès reproducteur, fournira une forte évidence que la signification de l'hybridation introgressive mitochondriale n'est pas neutre mais plutôt de nature sélective.

IV. Méthodologie

Les analyses génétiques incluront: (1) la caractérisation d'une population de *S. fontinalis* introgressés possédant le génome mitochondrial de *S. Alpinus* et (2) la détermination de la distribution géographique des populations de *S. fontinalis* introgressés

Caractérisation d'une population de *S. fontinalis* possédant le génome mitochondrial de *S. alpinus*.

Une population de *S. fontinalis* introgressés a été découverte dans le lac Alain (Québec) au cours d'une étude macrogéographique sur la variation génétique des ombles de fontaine de l'est de l'Amérique du Nord. Cette population a été caractérisée génétiquement et morphologiquement en utilisant des analyses d'ADNmt, des allozymes nucléaires ainsi que des mesures morphométriques. Afin de tester correctement l'étendue de l'introgresion

nucléaire ou mitochondriale chez les ombles du lac Alain, ces poissons ont été comparés à des ombles de fontaine non introgressés (Grand lac du Nord) et à des ombles chevaliers typiques (lac Rond) de la région de Québec. Étant donné que l'ADNmt est homoplasmique, c'est-à-dire que toutes les molécules sont identiques dans un organisme donné, il est plus pratique d'utiliser un tissu à partir duquel il est facile d'extraire l'ADN (Gyllensten et Wilson, 1987). Ainsi, le foie a été utilisé comme source d'ADN afin de caractériser génétiquement les populations introgressées.

Emplacement et description des échantillons

Les ombles de fontaine ont été échantillonnés dans le lac Alain et dans le Grand lac du Nord. Ces deux lacs sont situés dans le système de la rivière Portneuf. Les ombles chevaliers originant du Labrador ont été échantillonnés dans le lac Rond et proviennent également d'une pisciculture locale.

Analyses des allozymes

Des électrophorèses de protéines provenant d'homogénats de foie ont été effectuées sur de l'acétate de cellulose. Nous avons examiné les poissons pour plusieurs loci reconnus pour être polymorphiques chez l'omble de fontaine et l'omble chevalier (Hammar et al., 1991). Quatre enzymes représentant six loci ont été utilisées pour différencier les ombles de fontaine et les ombles chevaliers incluant: l'isocitrate déshydrogénase, la lactate déshydrogénase, la sorbitol déshydrogénase et la superoxyde dismutase. Les allèles ont été identifiés par leur migration électrophorétique respective sur les gels.

Caractérisation de L'ADN mitochondrial

L'ADN total a été extrait des individus du lake Alain, lake Rond et le Grand lac du Nord comme décrit antérieurement par Bernatchez et al., (1992). Un fragment de 2,5 Kb. du génome mitochondrial situé dans la région ND-5/6 a été amplifié par RPC (réaction polymérase en chaîne) . L'ADN amplifié a été digéré avec trois enzymes de restriction (*Ava*I, *Hae*III et *Hinc*II) qui génèrent des patrons de fragments diagnostiques dans le segment ND-5/6 entre les ombles de fontaine et les ombles chevaliers.

Détermination de la distribution géographique des populations de *S. fontinalis* introgressés.

Cette portion de l'étude a été entreprise afin d'examiner l'étendue de l'introgression de l'ADN mitochondrial (ADNmt) dans les populations d'ombles de fontaine et de déterminer la distribution géographique de ces populations dans les bassins de la région de la Côte-Nord du sud-est du Québec.

Emplacement et description des échantillons

L'effort initial d'échantillonnage s'est concentré autour du tributaire de la rivière Rocheuse du système de bassins Portneuf, où la population du lake Alain a été pour la première fois découverte. Les ombles de fontaine ont été échantillonnés dans plusieurs lacs. L'échantillonnage s'est étendu jusqu'à des populations provenant de plusieurs bassins

avoisinants incluant les Escoumins, Sault-au-Mouton, Sault-aux-Cochons, Laval, Betsiamites et aux Anglais rivers.

Allozymes et caractérisation de l'ADNmt

L'identification des ombles par électrophorèse des allozymes et la caractérisation de l'ADNmt ont été accomplies comme décrit précédemment pour la population du lake Alain.

B. Analyses physiologiques

Les analyses physiologiques incluront: (1) Caractérisation des fonctions enzymatiques et mitochondriales chez des populations de *S. fontinalis* introgressés et non introgressés acclimatés à basses températures. (2) Caractérisation des fonctions au niveau de l'animal entier en déterminant le registre aérobie pour l'activité et la performance de nage chez des populations de *S. fontinalis* introgressés et non introgressés.

Caractérisation des fonctions enzymatiques et mitochondriales chez des populations de *S. fontinalis* introgressés et non-introgressés.

La majorité des enzymes impliquées dans la phosphorylation oxydative (chaîne de transport des électrons et ATP synthase), comme la cytochrome oxydase, sont partiellement encodées par le génome mitochondrial. Par contre, la malate déshydrogénase est strictement encodée par le génome nucléaire mais est fonctionnelle dans les mitochondries. Cette enzyme sera par conséquent mesurée et utilisée comme contrôle étant donné qu'aucune différence dans son activité ou sa sensibilité thermique n'est envisagée entre les populations de *S. fontinalis*.

Le muscle rouge a été choisi comme tissu afin de déterminer les fonctions enzymatiques et mitochondriales. Les fonctions du muscle rouge peuvent être intégrées d'un niveau d'organisation biologique à l'autre, jusqu'à leurs manifestions finales au niveau de l'organisme entier où l'on peut mesurer la capacité de nage soutenue. L'interprétation d'un niveau organisationnel à l'autre peut être plus facile dans le muscle rouge que dans le foie, car dans ce dernier les liens entre les fonctions cellulaires et les fonctions de l'animal entier sont plus difficiles à discerner. De plus, le muscle rouge contient beaucoup de mitochondries comparativement au muscle blanc.

La sensibilité thermique d'une réaction enzymatique est un bon indicateur de la nature structurelle de l'enzyme. Généralement, des valeurs de Q_{10} faibles correspondent à des enzymes qui ont des structures rigides alors que des valeurs de Q_{10} élevées signifient que les enzyme possèdent des structures relativement flexibles. Les espèces adaptées au froid posséderaient des structures enzymatiques flexibles. La sensibilité thermique est une mesure des effets d'un changement de température sur les taux de réaction et peut être exprimée en calculant une valeur de Q_{10} (Hochachka and Somero, 1984). Le Q_{10} est défini comme:

$$Q_{10}= (k_2/k_1)^{10/(T°2-T°1)}$$

où k_1 et k_2 sont les taux de vitesse de la réaction aux températures $T°_1$ et $T°_2$ respectivement.

Origine des poissons et conditions d'acclimatisation

Les ombles de fontaine introgressés ont été échantillonnés dans le lac Alain et transportés vivants à l'INRS-oceanologie (Rimouski), où ils ont été transférés dans un bassin contenant de l'eau circulante. Les ombles de fontaine non introgressés ont été obtenus du

système Laval river et maintenus dans un bassin séparé, mais similaire à celui des ombles de fontaine introgressés. Étant donné que les différences dans les performances physiologiques mitochondriales ont été évaluées à partir de l'origine de L'ADNmt, il était important de maintenir le plus de paramètres environnementaux constants entre les deux groupes de poissons. Ainsi, la photopériode, la nourriture et la température de l'eau étaient identiques pour les deux groupes de poissons.

Isolement des mitochondries

Les mitochondries ont été isolées du muscle rouge en utilisant la technique décrite dans Chamberlin et al. (1991).

Détermination de l'efficacité catalytique des enzymes respiratoires mitochondriales.

Afin de tester *in vitro* les effets d'un changement de température (Q_{10}) sur la réponse thermique des enzymes respiratoires mitochondriales codées par l'ADNmt et d'une enzyme mitochondriale non-codée par l'ADNmt, les taux de réaction de la cytochrome C oxydase et de la malate déshydrogénase seront déterminés à différents températures.

Les essais enzymatiques seront effectués à 6, 12 et 18 °C en utilisant un spectrophotomètre connecté à un bain thermorégulé. Les activités enzymatiques maximales (V_{max}) de la cytochrome oxydase (CO) (Blier and Guderley, 1988) et de la malate déshybrogénase (Gerrits, 1994) seront déterminées. Les activités enzymatiques seront exprimées en unités par mg de protéine, où une unité équivaut à une µmole de substrat convertie en produit par minute.

Détermination de l'oxydation mitochondriale

Les mesures de consommation d'oxygène seront effectuées sur une suspension de mitochondries isolées du muscle rouge en utilisant des respiromètres connectés à des bains thermorégulés d'eau circulante, comme décrit précédemment dans Chamberlin et al. (1991). Des concentrations saturantes de substrat seront ajoutées afin d'estimer le stade 3 de la respiration mitochondriale, comme défini par Chance and Williams (1956). Le stade 3 mitochondrial procure des indices de la situation *in vivo* car il représente approximativement le flux maximal possible pour un substrat donné dans un tissu performant au maximum. Les substrats utilisés seront représentatifs des carburants métaboliques impliqués dans le métabolisme respiratoire. Ceux-ci incluent le pyruvate, le succinate, la glutamine, le malate et le palmitate. Les taux respiratoires seront exprimés en nmoles d'O_2 consommées par minute par mg de protéine mitochondriale.

Détermination des protéines

Les protéines seront déterminées par la méthode de Bradford (1976) en utilisant de l'albumine de sérum bovin comme standard. Les protéines mitochondriales et tissulaires seront déterminées en mesurant la différence entre la concentration protéique dans le milieu d'isolation et celle dans la suspension mitochondriale.

Caractérisation des fonctions au niveau de l'animal entier en déterminant la consommation d'oxygène durant la nage chez des populations de *S. fontinalis* introgressés et non introgressés.

La relation linéaire entre la consommation d'oxygène et la vitesse de nage permettera l'évaluation de la capacité de nage chez des populations de *S. fontinalis*. La performance de

nage sera évaluée en se basant sur plusieurs paramètres de capacité de nage, comme il a été décrit pour le corégone par Bernatchez and Dodson (1985). Ces paramètres incluent la vitesse de nage critique, le métabolisme actif et le registre métabolique pour l'activité. La vitesse de nage critique sera déterminée pour chaque poisson. Cette dernière est définie par Brett (1964) comme:

$$C=V + (t°\Delta t^{-1})\Delta v$$

où C est la vitesse de nage critique (cm^{-1}), Δt représente la période de temps prescrite (min), Δv est l'augmentation de la vitesse (cm^{-1}), t est le temps que le poisson a nagé à la dernière vitesse (min), et v est la plus grande vitesse maintenue pour la période d'augmentation prescrite (cm^{-1}).

Le métabolisme actif (ou la consommation active d'oxygène) corresponds à la consommation d'oxygène à la vitesse de nage critique. Cette consommation d'oxygène est prédite par l'équation de la régression entre la consommation d'O_2 et la vitesse de nage. Finalement, le registre métabolique pour l'activité ou le coût aérobie net de la nage est obtenu en soustrayant le taux métabolique standard du taux métabolique actif. Le taux métabolique standard est obtenu par extrapolation, à une vitesse de nage nulle, de la relation entre la consommation d'oxygène et la vitesse de nage.

Origine des poissons et conditions d'acclimatisation

Des ombles de fontaine introgressés et matures ont été échantillonnés dans le lac Alain et tranportés vivants à l'INRS-océanologie (Rimouski), où ils ont été maintenus dans des bassins jusqu'à la fin novembre, moment de la provocation du frai. Également, des

ombles de fontaine non introgressés ont été échantillonnés dans la Laval river et conservés jusqu'à la provocation du frai. Les oeufs ont été conservés jusqu'au moment de l'éclosion dans des plateaux situés dans un bassin à l'intérieur duquel traverse un courant d'eau. Après l'éclosion, les plateaux ont été retirés du bassin. La photopériode, la nourriture ainsi que la température de l'eau étaient identiques pour les deux groupes de poissons.

Mesures de la consommation d'oxygène

Les mesures de consommation d'oxygène seront effectuées à 6, 12 et 18°C en utilisant des respiromètres de type Blazka (Waiwood et Beamish, 1978). Les électrodes mesurant la consommation d'oxygène seront connectées à un ordinateur. Ceci permettera de prendre des mesures directes et simultanées. Chaque poisson sera acclimaté à la température d'essai et pesé avant de mesurer sa consommation d'oxygène. Les mesures de consommation d'oxygène durant la nage seront effectuées lors de l'augmentation de la vitesse du courant de 5 cm s^{-1} et ce, jusqu'au point de fatigue (lorsque le poisson est incapable de nager). Les poissons pourront nager durant trente minutes pour chaque augmentation de vitesse. Les taux de respiration seront exprimés en mg d'oxygène consommé par minute.

V. Résultats et interprétation

Une population d'ombles de fontaine (*Salvelinus fontinalis*) découverte dans le lac Alain, Québec, a été caractérisée et par la suite utilisée comme modèle afin de tester la

signification évolutive de l'introgression de l'ADNmt. Cette population possède le génome mitochondrial de l'omble chevalier (*S. alpinus*) malgré l'absence de cette espèce dans ce cours d'eau. Cependant, les individus introgressés sont identiques génétiquement aux ombles de fontaine purs. Ceci est basé sur des analyses d'allozymes du génome nucléaire et des caractéristiques morphologiques et méristiques. Donc, ces résultats ont permis d'établir que cette population d'ombles de fontaine était introgressée avec le génome mitochondrial de l'omble chevalier avec aucune apparence d'introgression nucléaire. De plus, ces résultats indiquent que l'haplotype d'ADNmt observé chez les ombles de fontaine du lac Alain est le résultat d'une ancienne introgression avec l'omble chevalier plutôt qu'un ancien polymorphisme ou une évolution convergente. Ces résultats ont aussi servi à démontrer que l'hybridation introgressive entre ces deux espèces peut avoir des effets significatifs et à long terme sur leur composition génétique.

L'étendue géographique des populations de *S. fontinalis* introgressés dans la région de la Côte-Nord de l'est du Québec a également été déterminée. Cette étude a montré que les populations de *S. fontinalis* sont restreintes seulement au tributaire de la Rocheuse river du système de bassins Portneuf. Ailleurs dans le système de bassins Portneuf et dans les bassins avoisinants, des populations de *S. fontinalis* purs et non introgressés peuplent les lacs. Les ombles chevaliers sont complètement absents de ces bassins. Ensemble, ces résultats montrent que l'évènement initial d'hybridation entre ces espèces est ancien et s'est probablement produit peu de temps après la recolonisation de la région par les deux espèces. À ce moment, les espèces auraient été en contact et les chances que les mécanismes d'isolement reproducteur se soient rompus auraient été élevées. Il est possible qu'une

combinaison de conditions biogéographiques couplée à une sélection positive pour l'introgression de l'ADNmt aient conduit à la distribution actuelle des *S. fontinalis* introgressés dans l'est du Québec.

Comme première étape de l'approche expérimentale, l'efficacité catalytique ainsi que la sensibilité thermique (Q_{10}) de la cytochrome C oxydase (CCO) ont été déterminées dans des *S. fontinalis* introgressés et non introgressés et comparées à celles de la malate déshydrogénase (MDH). Des réponses différentes entre les groupes de poissons pour l'efficacité catalytique et les valeurs de Q_{10} ont été pricipalement observées pour la CCO, mais non pour la MDH. Ceci suggére une influence du génome mitochondrial, étant donné que la CCO est partiellement encodée par l'ADNmt. Ce résultat est consistant avec la présomption que le génome mitochondrial, comme tel, influence la structure enzymatique, et donc la fonction enzymatique. En se basant sur ces différences enzymatiques, la prédiction au niveau de la fonction mitochondriale est que les *S. fontinalis* introgressés posséderaient des mitochondries qui fonctionneraient mieux à basses températures. En effet, les *S. fontinalis* introgressés montraient une capacité mitochondriale accrue à basses températures pour ce qui est de l'oxydation du malate et du succinate. Des différences dans l'oxydation pyruvate ont aussi été observées à des températures plus élevées, ainsi que des différences dans les valeurs de Q_{10}, ce qui pourrait réfléter une hausse de l'efficacité de la CCO. Ensemble, ces résultats fournissent une évidence de l'influence de l'ADNmt sur la structure et la fonction des enzymes ainsi qu'une influence non neutre au niveau de la fonction mitochondriale.

La capacité aérobie et la performance de nage des poissons ont été évaluées en utilisant des paramètres énergétiques déterminés par repirométrie natatoire. Les *S. fontinalis*

introgressés avaient un registre métabolique pour l'activité aérobie semblable aux *S. fontinalis* non introgressés à basse tempèrature. Des analyses ultérieures du registre métabolique ont démontré que le potentiel pour satisfaire le métabolisme nutritionnel est également semblable.

Les résulats de cette étude démontrent, pour la première fois, que la variation au niveau de l'ADNmt résultant d'évènements introgressifs peut être fonctionnellement inportante aux niveaux moléculaire et cellulaire, et probablement sélectivement maintenue dans des populations naturelles. Ces données sont d'un intérêt particulier pour la biologie des populations et l'évolution car la variation de l'ADNmt est considérée traditionnellement comme ayant des conséquences neutres.

CHAPTER ONE

Introduction

Natural hybridization between closely related fish species occupying different thermal niches can occur in ranges where distributions overlap. One rare consequence of natural hybridization is introgression, where novel genetic material or genes from one species are transferred to the other species (Verspoor and Hammar, 1991). The evolutionary significance of introgressive hybridization in fish is not well understood. There may be selective advantages to possessing genes from another species that contribute to the fitness of introgressed individual.

In the past, selectionist hypotheses of this type have been proposed but, they have never been specifically tested. Interspecific gene transfer, as occurs for introgression, has been argued to be a neutral phenomenon and this view has prevailed (Dowling and Hoeh, 1991). The acceptance of neutrality without further considering adaptability has hampered our understanding of the relative roles of deterministic and stochastic processes as driving forces of gene exchange among different taxa (Arnold, 1992). In order to strictly test the selectionist hypothesis an experimental approach that substantiates predictions of "superior" responses at different levels of biological organization is required (Powers et al., 1991; 1993).

The present research will serve to develop a better understanding of the evolutionary significance of introgressive hybridization in fish. The primary focus of my research will be to investigate the influence of introgressed mitochondrial genes on physiological performance at different levels of biological organization. Demonstration of a "superior" performance in

introgressed populations will provide strong evidence that introgressive hybridization is of a selective nature.

1.1 Natural Hybridization and Introgression

The ability of reproductively distinct populations and species of fish to interbreed or hybridize and produce viable offspring is now well established. Compared to other vertebrate groups, fish and particularly freshwater fish have a relatively high occurrence of hybridization in nature (Campton, 1987). The ability of fish to attain rather high levels of interspecific hybridization is primarily attributed to external fertilization. Other factors thought to contribute to the elevated capacity of fish as a group to hybridize are: unequal numbers of parental species in sympatric fish populations i.e. lack of conspecific mates, competition for limited spawning sites and secondary contact enhanced by human intervention, particularly introductions (Campton, 1987; Billington and Hebert, 1991). The recency of most freshwater habitats may have also heightened the ability of freshwater fish to hybridize (Hubbs, 1955). Most fish species in the Northern hemisphere evolved during the Pleistocene and in those species, isolating mechanisms such as different spawning times may not have had time to develop completely (Billington and Hebert, 1991).

Within the salmonid group of fish, there is little information on natural hybridization among species (Hubbs, 1955; Verspoor and Hammar, 1991). When it has occurred, interspecific hybridization has been between congeneric salmonids. Natural hybridization has been reported for the genus *Oncorhynchus* (Allendorf and Phelps, 1980; Campton and Johnston, 1985; Marnell et al., 1987; Bartley et al., 1990; Bartley and Gall, 1991; Busack and Gall, 1991) and *Coregonus* (Vuorinen, 1988). Within the genus *Salvelinus*, natural hybrids

are known to occur between Arctic char (*S. alpinus*) and brook trout (*S. fontinalis*) (Hammar et al., 1991), as well as between Arctic char and lake trout (*S. namaycush*) (Hammar et al., 1989; Wilson and Hebert, 1993). Although from this review, it would appear that hybridization in salmonids is a common and well-understood event, oftentimes hybridization reports have been of a single occurrence (Bartley et al., 1990) or little has been known of the geographical extent of the hybridizing population (Hammar et al., 1989; 1991).

The Arctic char, *S. alpinus* has the most northerly distribution of any freshwater fish occurring south as far as eastern Quebec (Scott and Crossman, 1973). In contrast, *S. fontinalis* has a more southern distribution, extending south into the United States and north along the Hudson Bay coast (Scott and Crossman, 1973). Despite the fact that these species essentially occupy different thermal niches, natural hybridization has been reported between these species at the extremes of their respective ranges where their distributions overlap (Hammar et al., 1991).

One consequence of natural hybridization is introgression, which involves the transfer of novel genetic variants into a population or species by the other (see Verspoor and Hammar, 1991). As long as the hybrid progeny are fertile, hybrids back-crossing into one or the other parental populations may lead to the transfer of genes between taxa. Introgressive hybridization may, therefore, give rise to temporary or permanent changes in the gene pools of the hybridizing populations depending on the degree of reproductive separation and assortative mating (Verspoor and Hammer, 1991). Although potentially an important pathway for gene flow between species, the occurrence of introgression in fish is relatively rare and its impact on genetic variation is still poorly understood.

In order to assess the actual extent of introgressive hybridization among hybridizing populations, the direct analysis of genes in the hybridizing population is required (Campton, 1987; Verspoor and Hammar, 1991). Since there is potential transfer of genes from both nuclear DNA and mtDNA, both genomes must be analyzed for diagnostic fixed differences (Campton, 1987). The conventional segregation of nuclear DNA makes nuclear introgression relatively easy to detect by electrophoretic methods when the two parental species are completely fixed for different alleles (Campton, 1987). In contrast, mtDNA is strictly maternally inherited so that the analysis of mtDNA will only reveal the identity of the maternal parent. This property preludes the use of mtDNA analysis as the only technique for detecting introgression. Only by combining mtDNA and allozyme analysis can the extent and directionality of gene transfer be determined.

1.2 Importance of Introgression

The transfer and fixation of mtDNA between congeneric populations may have important evolutionary consequences. To date, investigations into the selective importance of introgression have not been undertaken. However, the possibility that a "selective advantage" is incurred from the donor species' genome has been suggested. Dowling et al. (1989) suggest that variation in mtDNA haplotypes resulting from introgression observed between two river drainages was due to geographically variable patterns of selection against different hybrid and backcross combinations. Introgressed hybrids may be more fit by virtue of containing genes from another species, especially if that species is adapted to more extreme environmental conditions, for example colder temperatures.

1.3 Experimental approach for studying nuclear genetic variation

The potential adaptive role of nuclear genetic variation of enzyme synthesizing loci has undergone extensive investigation. The evolutionary significance of protein polymorphism has been a topic of debate centered around two contrasting views: the selectionist view and the neutralist view (Clarke, 1973). Selectionists assert that natural selection maintains protein polymorphisms, whereas those of the latter persuasion argue that the vast majority of such variation is selectively neutral. A few evolutionary biologists have developed and used an experimental approach that allows the evaluation on a theoretical basis of the selectionist/neutralist controversy (Clarke, 1975; Powers et al., 1991). The experimental strategy developed so far involves the biochemical study of allelic isozymes so that predictions of differential responses can be made at different levels of biological organization. As a first step, this strategy requires a detailed biochemical and physiological study of the allelic isozymes. Based on the nature of the differences found, the function of the enzyme, and the ecology of the organism, a selective factor can be postulated and a hypothesis generated that establishes a mechanistic link between the selective factor and the gene product. Finally, the hypothesis is tested by experimentally manipulating the environment to produce a predictable response. Some examples that reflect this approach include: Alcohol dehydrogenase from *Drosophila* where kinetic differences between allelic isozymes are associated with differences in survivorship and developmental time (Daly and Clarke, 1981; Vigue et al., 1982; Dorado and Barbancho, 1984); Aminopeptidase from *Mytilus* where activity differences between allozymes are associated with improved osmoregulation and fitness (Hilbish and Koehn, 1985); Several

enzymes from *Fundulus* where differences in the kinetic and other biochemical properties of allelic isozymes (Place and Powers; 1984; Ropson and Powers 1988; 1989; Van Beneden and Powers, 1989) were used to predict and subsequently establish significant differences in metabolism, oxygen transport, swimming performance, developmental rate and relative fitness (DiMichele and Powers, 1982; 1991; Paynter et al., 1991). These examples illustrate the use of an experimental approach to resolve the neutralist/selectionist controversy for nuclear genetic variation. So far, such an approach has not been applied to study the evolutionary significance of mitochondrial genetic variation.

1.4 MtDNA genome

MtDNA is a circular molecule usually ranging in size from 16 000 to 18 000 base pairs (bp) in fish (see Gyllensten and Wilson, 1987). The rate of nucleotide substitution in the mtDNA of higher vertebrates is approximately 5-10 times that of the nuclear genome (Moritz et al., 1987). MtDNA sequence divergence for congeneric fish generally ranges from 1.4%-16% (Billington and Hebert, 1991). A notable feature of mtDNA is that it is maternally inherited and not subject to recombination (Avise and Vrijenhoek, 1987). Generally, mtDNA is homoplasmic, that is, all the molecules are identical in an organism, meaning that any tissue can be used as a source (Gyllensten and Wilson, 1987) .

One of the many properties of mtDNA that distinguish it from nuclear DNA is its variability in gene content (Ferguson and Allendorf, 1991). Generally, the vast majority of genes, even those required for mitochondrial function, are encoded in the nucleus. In contrast, the mtDNA molecule is relatively conserved in content and codes for 2 ribosomal RNA genes, 22 transfer RNA genes, and 13 protein genes that code for subunits of enzymes

functioning in electron transport or ATP synthesis (Anderson et al., 1981; Chomyn et al., 1985; Chomyn et al., 1986). While 90% of mitochondrial proteins are encoded by the nuclear genome, the remaining 10% are very important as they encode for subunits of enzymes central to cell respiration.

1.5 Consequences of Gene Mutations

One focus of molecular evolutionary studies is on how gene mutations, and the consequent amino acid substitutions in the enzymes they encode, may alter the metabolism of organisms. It is this alteration in phenotype that affects interactions with the environment and ultimately, adaptation to specific niches (Hilbish and Koehn, 1985). Since most fish are nearly ideal ectotherms (Hazel and Prosser, 1974), temperature is a major constraint on metabolism. Temperature has a direct and dramatic effect on enzyme reaction rates so that their metabolic rate is highly responsive to changes in environmental temperature. A variety of mechanisms have been described to explain partial or complete metabolic adaptation in fish after exposure to altered thermal conditions (Hazel and Prosser, 1974; Hochachka and Somero, 1984 for review). While thermal adaptation in fish has been demonstrated over an evolutionary time scale (Graves and Somero, 1982; Crockett and Sidell, 1990) as well as an acclimatory time scale (several weeks usually) (Johnston and Harrison, 1985), the mechanisms involved are not instantaneous. In fact, adjustments in metabolic capacity reflect fundamental genetic differences reflected in the enzyme systems of warm- and cold-adapted (-acclimated) ectotherms (Hochachka and Somero, 1984).

One enzymatic mechanism recognized to be fundamental and of great importance in the evolutionary adaptation to temperature is changes in the catalytic efficiencies of

enzymes (see Hochachka and Somero, 1984). A consistent trend is that enzymes of cold-adapted species have higher catalytic efficiencies than those of warm-adapted species, due to the relative flexibilities of the enzymes. Catalytic efficiency is defined as how rapidly the enzyme can convert substrate to product as expressed on a per enzyme molecule basis. One expression for catalytic efficiency is the substrate turnover number, often measured as moles of substrate converted to product per mole enzyme per unit time (generally one minute) (Lehninger et al., 1993). Determination of this parameter entails measurement of enzyme function at its maximal possible velocity (V_{max}). The basis for differences in enzyme efficiency between species adapted to different thermal environments is related to the change in enzyme conformation which accompanies catalysis (Hochachka and Somero, 1984). The flexibility in enzyme structure is an obligatory part of binding events between the binding site and substrate, and is also important for the formation of the activated enyzme-substrate complex. Because changes in enzyme conformation entail the breaking and formation of weak chemical bonds, there are energy changes associated with conformational changes. One model that has been proposed to account for different conformational energy expenditures during catalysis in enzyme systems from organisms adapted to different temperatures involves the use of different numbers (or types) of weak interactions to stabilize protein conformation (Hochacka and Somero, 1984).

The inherent flexibility of enzymes is conferred by the presence of weak bonds such as hydrogen bonds, charge-charge interactions, and van der Waals interaction, all of which serve to stabilize the higher orders of protein structure (Hochachka and Somero, 1984). Presumably a highly flexible enzyme, stabilized by few weak bonds, will readily undergo

the conformational transitions during catalysis and, thus, will require less energy input to acheive catalysis. Enzymes of low body temperature species gain their heightened catalytic efficiency via possession of a more flexible structure. Hochacka and Somero (1984) suggest that over an evolutionary time span, during which rebuilding of the amino acid sequence of enzymes is possible, adjustments in the catalytic efficiency through weak bonds are fabricated in most enzymes.

Some of the mechanisms for enhancing thermal stabilities of proteins have been studied by Argos et al. (1979). Their study showed that genetically, only a singe base pair was involved for structurally transforming a mesophilic protein into a thermophilic protein. Thus, in terms of genetic structure, as well as amino acid composition, the building of thermophilic efficient enzymes appears a relatively simple evolutionary task. Nucleotide differences, as observed in the mtDNA genome of closely related species, may be sufficient to influence the catalytic efficiency of the respiratory enzymes they code for and, therefore, be a determining factor for defining the limits of a species' thermal habitat or the fitness of an organism in a given thermal regime.

1.6 Respiratory Enzymes (Oxidative Phosphorylation)

Mitochondria are the major site of ATP generation, which is needed to meet the energetic requirements of an organism. When the substrates of fuels, such as lipids, carbohydrates or proteins are catabolized inside the mitochondria, energy-rich molecules, NADH and $FADH_2$ are formed which contain a pair of electrons having high energy transfer potential (Stryer, 1988). These electrons are donated to oxygen, and a large amount of free energy is liberated, which is used to generate ATP. Oxidative phosphorylation is a process in

which ATP is formed as electrons are transferred from NADH or $FADH_2$ to O_2 by a series of electron carriers (Stryer, 1988). The step by step transfer of electrons through these enzymes leads to the pumping of protons out of the mitochondrial matrix. The pH gradient and membrane potential that are created in this manner constitute a proton motive force used to drive the synthesis of ATP, as the protons flow back to the mitochondrial matrix through ATP synthetase (Ragan et al., 1987).

1.7 Red Muscle

Highly aerobic tissues are necessarily dependent on the performance of mitochondria for the production of ATP. One such tissue in fish is red muscle. In fish, red fibres are frequently concentrated as a discrete region of "red muscle" along the lateral line which may constitute as much as 29% of the muscle mass (Johnston, 1981). In red muscle, mitochondrial volume density is high (25%-38%) (Johnston, 1981). High mitochondrial volume densities are necessary to meet the ATP demands of sustained swimming activity. Red muscle in fish functions predominantly under aerobic conditions and is responsible primarily for sustained swimming activity. In accordance to this, red muscle must be extensively vascularized to provide an adequate supply of oxygen and nutrients.

Relatively little is known about red muscle metabolism. Carbohydrates, in general, are poorly utilized in red muscle of salmonids (Lin et al., 1979), but pyruvate is readily oxidized by mitochondria from fish red muscle (Blier and Guderley, 1993; Moyes et al., 1989). Lipids were one of the first metabolic fuels identified as an important energy source for red muscle (Bilinski, 1974; Van den Thillart, 1986). High rates of fatty acid oxidation by

isolated mitochondria (Ballantyne et al., 1989; Moyes et al., 1989; Chamberlin et al., 1991) indicate a high capacity to utilize lipids in red muscle. Amino acids from proteins are also known to be important energy source. Recently, high rates of glutamine oxidation in isolated red muscle mitochondria (Chamberlin et al., 1991) have indicated the catabolic potential of glutamine in this tissue. The rates of substrate oxidation in red muscle of fish and, therefore, the capacity of mitochondrial performance may be enhanced by respiratory enzyme systems originating from cold-adapted organisms, allowing them superior swimming capacity. Generally, fish acclimated to low temperatures display an increased aerobic swimming capacity (Guderley and Blier, 1988; Guderley, 1990).

1.8 Summary

The evolutionary significance of mitochondrial introgression in fish is not known. It is probable that the mitochondrial genome acquired from the donor species imparts a selective advantage to introgressed individuals, particularly if the donor species is adapted to a more extreme environment, for example one which is colder. The experimental approach, originally developed to resolve the selectionist/neutralist controversy for nuclear protein polymorphism can be applied to study the evolutionary significance of mitochondrial genetic variation generated through introgressive events. The mitochondrial genome partly encodes for respiratory enzymes important for the production of ATP, which is needed to meet the energy demands of the organism. Enzymes of cold-adapted species have enhanced structural flexibilities which are genetically determined and allow the enzymes to function better at cold temperatures than those of warm-adapted species. Fish species introgressed with the

mitochondrial genome of a cold-adapted species may, therefore, have respiratory enzymes which function better at low temperatures. Since respiratory enzymes influence mitochondrial performance and ultimately enhance the performance of a tissue like red muscle, in fish, this may be reflected at the individual level in the swimming capacity of the organism. The swimming capacity may be a determining factor for the fitness of the individual and, therefore, introgressed fish possessing the mitochondrial genome of a colder adapted species may display a selective advantage over typical non-introgressed individuals at cold temperatures.

1.9 Hypothesis, Approach and Outline

1.9.1 Research Hypothesis

The hypothesis to be tested is that introgressed brook char *(Salvelinus fontinalis)*, possessing the mitochondrial genome of the more northerly distributed Arctic char species (*S. alpinus)*, have a physiological advantage at low temperatures compared to non-introgressed brook char possessing *S. fontinalis* mitochondrial DNA.

1.9.2 Experimental Approach

The primary focus of this research will be to investigate the genetic influence of the mitochondrial genome on the physiological performance of *Salvelinus fontinalis* populations at low temperatures so that the evolutionary importance of mtDNA introgression can be clarified. The approach used will be a comparative one, involving the identification of both

genetic and physiological differences between introgressed *S. fontinalis* and non-introgressed *S. fontinalis*. A multi-level approach was also necessary for understanding and identifying the influence of the mitochondrial genome on the physiological performance of *S. fontinalis*. Such an approach focuses on the physiological function at different levels of biological organization (Table 1.1).

Briefly, genetically determined differences in the mtDNA genomes may be reflected at the molecular level by altering the tertiary subunit structure of mitochondrial respiratory enzymes that this genome partially encodes. These mitochondrial enzymes may display different catalytic efficiencies due to differences in flexibility imparted by subunit structure. At the cellular level of organization, since cellular respiration is dependent on mitochondrial respiration and, thus, the respiratory enzymes, the functional efficiency of mitochondria may be affected. Finally, an integration of function throughout the levels of organization, differentiated through an influence from the genome on protein subunit structure, may be apparent at the organismal level and manifested in the performance capacity of the organism.

Since mtDNA is generally homoplasmic, that is, all the molecules are identical in an organism, it is sometimes more convenient to use a tissue that is easily obtainable as a source of DNA (Gyllensten and Wilson, 1987). For this reason, liver tissue was used as a source of DNA to characterize introgressed populations. However, red muscle was chosen as the tissue for which enzyme and mitochondrial function were determined. Functional aspects of red muscle metabolism may be integrated from one organizational level to the next until it is finally manifested at the individual level by a measurable performance parameter, i.e. aerobic swimming capacity. Interpretation from level to level may be easier in red muscle, rather

than liver, where the link from cellular function to organismal function is much harder to discern. Red muscle is also a good source of mitochondria, as compared to white muscle

Introgressed populations are expected to have a superior performance capacity at low temperatures due to the benefits procured from the mitochondrial genome of a northern species throughout all these levels of biological organization. Demonstration of superior performance and, therefore, probable superior fitness at low temperature in an introgressed *S. fontinalis* population will provide strong evidence that the significance of mitochondrial introgressive hybridization is not neutral but rather of a selective nature.

1.9.3 Thesis Outline

In Chapter Two, mtDNA introgression by *S. alpinus* is characterized in a *S. fontinalis* population originating from Lake Alain in eastern Quebec, Canada. Individuals from this populations are characterized with respect to morphological and meristic characteristics, and to both nuclear and mitochondrial genomes by comparing introgressed *S. fontinalis* with non-introgressed *S. fontinalis* and *S. alpinus* individuals.

Chapter Three presents the geographical extent of introgression for *S. fontinalis* populations in the region of eastern Quebec where the original introgressed population was first discovered. Populations originating from the same drainage basin as the Lake Alain population, as well as those from neighbouring drainages are evaluated for introgression by analyzing both the mitochondrial and nuclear genomes.

In Chapter Four, the physiological performance at the molecular and sub-cellular levels of biological organization is evaluated in introgressed and non-introgressed *S.*

fontinalis individuals. The structural flexibility of a mitochondrial enzyme, cytochrome C oxidase, is determined by examining the effect of changes in temperature on enzyme activity. The influence of enzyme structure on mitochondrial function is assessed by following the thermal sensitivity of O_2 consumption by isolated red muscle mitochondria for different metabolic substrates.

In Chapter Five, the aerobic capacity at the individual level of biological organization is determined from energetic parameters obtained from swimming fish. The metabolic scope for aerobic activity and swimming performance are evaluated and compared between introgressed and non-introgressed *S. fontinalis*.

Finally in Chapter Six, conclusions and future directions for this research are presented. General conclusions are drawn from each chapter and the importance of the data, as well as directions for future research, are discussed.

Table 1.1. Multi-level approach for investigating genetic influences on the physiological performance of *S. fontinalis* populations.

Level of Organization	***Process Involved***	***Differences attributed to mtDNA***	***Parameter Determined***
Macro-Molecular	Mitochondrial enzyme function	Enzyme subunit structure	Mitochondrial enzyme activities
Sub-Cellular	Mitochondrial function	Enzyme subunit structure	Mitochondrial O_2 consumption
Organism	Swimming performance	Enzyme subunit structure	Swimming efficiency

CHAPTER TWO

Introgression and fixation of Arctic char (*Salvelinus alpinus*) mitochondrial genome in an allopatric population of brook char (*Salvelinus fontinalis*).[1]

2.1 Introduction

There is little consensus regarding the evolutionary significance of hybridization and introgression, although there is increasing evidence for their occurence. Traditionally, the transfer of genes across species boundaries has been thought to have little or no evolutionary importance (Mayr, 1963; Heiser, 1973). The main argument supporting this view is that introgressive hybridization among animal taxa is rare, and that documented cases leading to persistent, long-term incorporation of one species' genes into another are even rarer. Recent molecular and ecological studies, however, support the prevalence of introgressive hybridization in several species complexes and argue that even rare introgressive events may be more important than mutation as a source of new genetic variability within taxa (reviewed in Arnold, 1992).

Introgressive hybridization could be particularly important in fish, which, as a group, appear to hybridize fairly readily (Hubbs, 1955). Hybridization beyond the first generation has been reported for many species (reviewed in Verspoor and Hammar, 1991; Dowling *et al.*, 1989; Dowling and Hoeh, 1991; Carmichael *et al.*, 1993), suggesting the potential for introgression. One well-documented case of potential interspecific introgression is the natural hybridization between Arctic char (*Salvelinus alpinus* L.) and the brook char

[1] adapted from Can. J. Fish. Aquat. Sci. 52 (1): 179-185. 1995.

(*Salvelinus fontinalis* Mitchill) reported by Hammar *et al.* (1991). Protein electrophoresis detected repeated hybridization between these species in Northern Labrador. Furthermore, isozyme phenotypes suggested the presence of second-generation hybrids and/or backcrosses to both species. However, there was no evidence of persistent incorporation of genes from one species into the other, precluding any determination of the evolutionary impact of hybridization for these species.

In this chapter, we report the fixation of Arctic char mitochondrial DNA (mtDNA) in an allopatric population of brook char from an inland lake in Quebec, outside the present-day contact zone of the two species. This shows that introgressive hybridization as described by Hammar *et al.* (1991) can have significant and long-term effects on the genetic composition of brook char. This case of mitochondrial introgression represents one of the very few documentations of complete interspecific replacement of mitochondrial genome, and the first known occurence in salmonid fishes.

2.2 Material and methods

2.2.1 Sample description and location

Brook char were collected from Lac Alain (48°48'00", 69°35'30"), and Grand Lac du Nord (48°42'00", 69°40'00"), which are in the Portneuf River system (Figure 2.1), in the context of a macrogeographic survey of brook char in Eastern North America (Danzmann *et al.*, unpublished data). Sixteen fish were collected from Lac Alain in August 1992, and 32 additional fish were collected between January and July 1993. Arctic char do not occur in Lac Alain or its river system and the nearest reported population is approximately 50 km

away (Dumont, 1982; M. Brault, Ministère du Loisir, de la Chasse et de la Pêche, Québec, personal communication). Arctic char were collected from Lake Rond (48°15'00", 70°37'30"`) and obtained from a local pisciculture. Arctic char obtained from the pisciculture were of Fraser River (Labrador) origin. Liver was sampled from each fish and either processed immediately, or otherwise stored at -80 °C until further analysis.

2.2.2 Morphological identification

Upper and lower gill rakers were counted on the first left gill arch, and external features which are classically used to distinguish Arctic char and brook char were recorded on all freshly killed fish (Scott and Crossman, 1973). The diagnostic features examined were tail shape (square vs. forked), coloration of lower fins (presence/absence of a black stripe following the white border), presence/absence of vermiculations on the back, caudal and dorsal fins, and presence/absence of red spots with a blue halo on the sides of the body.

2.2.3 Allozyme Analysis

Protein electrophoresis was carried out on cellulose acetate using liver tissue homogenates as described by Hebert and Beaton (1989). Fish were screened at several loci known to be polymorphic for Arctic and brook char (Hammar *et al.*, 1991). Four diagnostic enzymes representing 6 loci were utilized to distinguish brook char from Arctic char and included: isocitrate dehydrogenase (IDH), 1.1.1.42; lactate dehydrogenase (LDH), 1.1.1.27; sorbitol dehydrogenase (SDH), 1.1.1.14; and superoxide dismutase (SOD), 1.15.1.1. Loci were designated as recommended by Shaklee *et al.* (1989). All enzymes examined were

resolved by using a Tris glycine buffer system of pH 8.5, and the staining recipes outlined by Hebert and Beaton (1989). Allelles were identified by their relative electrophoretic mobilities as measured from the gels. The most common allelle in the brook char population (Grand Lac du Nord) was assigned a standard mobility of 100.

2.2.4 Characterization of mitochondrial DNA

For the first 16 individuals analyzed from Lac Alain, mtDNA was extracted and purified from fresh liver by the rapid isolation method of Chapman and Powers (1984) with modifications of Danzmann *et al.* (1991a). Intact mtDNA was digested with eight restriction enzymes (*Acc*I, *Bam*HI, *Bcl*I, *Bst*EII, *Nco*I, *Nhe*I, *Pst*I, *Xba*I). Digested mtDNA fragments were separated on 0.8% agarose gel run overnight at 30 volts, visualized by UV irradiation after ethidium bromide staining, and photographed. All of these enzymes provided diagnostic fragment patterns between Arctic char and brook char throughout their distributions (Grewe *et al.*, 1990; Ferguson *et al.*, 1991; Danzmann *et al.*, 1991a; 1991b; Bernatchez and Danzmann, 1993; Wilson and Hebert, 1993; C. Wilson, unpublished results). Digested samples were subsequently compared against mtDNA digests from Québec Arctic char (Lake Rond) and brook char (Grand Lac du Nord).

Total DNA was extracted from the remaining 32 individuals of Lac Alain as described previously by Bernatchez *et al.* (1992). A 2.5 kb. fragment of the mitochondrial genome encompassing the ND-5/6 region was amplified by the polymerase chain reaction (PCR) with the primers published by Cronin *et al.* (1993). PCR reactions were those described by these authors and the amplification conditions consisted of a first denaturation

step of 95°C for 1 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 3 min. Amplified DNA was digested with three additional restriction enzymes (*Ava*I, *Hae*III, and *Hinc*II) which generated diagnostic fragment patterns in the ND- 5\6 segment between Arctic char and brook char. The DNA fragments were separated on 1.2% agarose gels, run for five hours at 85 volts, stained with ethidium bromide, and photographed with polaroid film under UV light.

2.3 Results

2.3.1 Morphological identification

Based on gill raker counts and external features, all Lac Alain fish collected were unambigiously identified as brook char. Lower and upper gill raker (LGR , UGR) counts (LGR range: 6-7, UGR range 10-12, total range; 15-19) were typical of brook char populations when compared to overall ranges reported for that species (LGR range: 4-7, UGR range 10-15, total range; 14-22) and for Arctic char (UGR range: 7-13, LGR range 12-19, total range; 19-32). All 48 individuals analyzed had a square tail, vermiculated patterns on the back, dorsal and caudal fins, a white border followed by a black stripe on lower fins, and red spots with a blue halo on the body sides. These morphological characteristics are all unambiguously typical of brook char.

2.3.2 Allozymes

Identification of char from Lac Alain (LA) was based on diagnostic fixed alleles between *S. alpinus* char (SA) and *S. fontinalis* (SF) at the IDH-3,4*, LDH-3*, SDH-1,2*, and

SOD* loci (Table 2.1) . Alleles at all loci in the char from Lac Alain were found to be identical to those characteristic of *S. fontinalis*, with no evidence of shared alleles with *S. alpinus*.

2.3.3 mtDNA characterization

Restriction analysis performed either over the entire molecule or on the ND 5\6 segment clearly showed that 100% of the 48 individuals characterized possessed only mitochondrial DNA of Arctic char origin (Table 2.2, Figure 2.2). Only one mtDNA genotype was observed in Lac Alain which matched the genotype of *S. alpinus* from Lac Rond. This is also the dominant genotype observed among many populations of Arctic char in Eastern North America (C. Wilson, unpublished data). This genotype was also similar to the dominant genotype reported for Icelandic char (Danzmann *et al.*, 1991b), with the exception of the fragment pattern for *PstI*. The large number of fish sampled from Lac Alain and the absence of any brook char mtDNA genotypes strongly suggest that brook char mtDNA has been eliminated from this population.

2.4 Discussion

Interspecific mtDNA exchanges have been reported in many animal groups including invertebrates (e.g. Powell, 1983; Solignac and Monnerot, 1986; Harrison *et al.*, 1987; Aubert and Solignac, 1990) amphibians (e.g. Spolsky and Uzzell, 1984; Lamb and Avise, 1986) reptiles (e.g. Wright *et al.*, 1983) and mammals (e.g. Tegelstrom, 1987; Lehman *et al.*, 1991).

In fish, interspecific transfer of mtDNA has been documented for several species (reviewed in Billington and Hebert, 1991). However, definite cases of introgression are rare and have generally been observed at very low frequencies (Billington *et al.*, 1988; Avise *et al.*, 1990; Bernatchez *et al.*, 1989; Bernatchez and Dodson, 1991; Wilson and Hebert, 1993). To our knowledge complete interspecific replacement of the mitochondrial genome in natural populations has been described in only one fish species complex, *Notropis cornutus/chrysocephalus* (Dowling *et al.*, 1989; Dowling and Hoeh, 1991). Therefore, the fixation of mitochondrial genome in Lac Alain brook char is unusual and, so far, unique in salmonids.

Alternative hypotheses to introgression might explain the distinct mtDNA composition of this population. First, ancestral polymorphic mtDNA may have been retained in different taxa since they diverged from a common ancestor. Second, identity with Arctic char mitochondrial genome may have resulted from convergent evolution. Generally speaking, it is often very difficult to distinguish which factor (or combination of factors) is responsible for the sharing of genetic variants in different taxa (discussed in Verspoor and Hammar, 1991). However, alternative hypotheses to introgression can easily be ruled out in the present case. MtDNA variation observed in all available studies (Grewe *et al.*, 1990; Ferguson *et al.*, 1991; Danzmann *et al.*, 1991a; 1991b; Bernatchez and Danzmann, 1993; Wilson and Hebert, 1993; C. Wilson, unpublished results) dealing with *S. alpinus* and *S. fontinalis* showed that no mtDNA genotypes are shared by both species over their range of distribution, which is inconsistent with a symplesiomorphic scenario. Secondly, net mtDNA

sequence divergence between genotypes of both species is relatively high, estimated at 3.0% by Grewe *et al.* (1990).

This implies that even the most parsimonious scenario would require a high number of homoplasic events to explain a confusion of mtDNA variants in brook char from Lac Alain with those of Arctic char, making the hypothesis of convergence very improbable. Therefore, there is no doubt that the mtDNA genotype observed in all brook char from Lac Alain has been incorporated through introgression with Arctic char. Because Arctic char have never been found in any lake of the Portneuf River system, despite intensive sport fishing and biological surveys conducted over the past 30 years, it is most likely that these introgressive events are ancient, and that their effect has persisted for a long time. As such, the present case clearly illustrates that introgressive hybridization may have a permanent effect on the genetic makeup of animal species. In Chapter three, a survey to verify if introgressed brook char are also present in other river systemes is undertaken.

The fact that fish from Lac Alain were otherwise indistinguishable from brook char based on morphological and allozyme criteria substantiates that nuclear introgression has not occurred or has long been diluted. Natural hybridization between sympatric Arctic char and brook char populations in Labrador has revealed both morphological and nuclear intermediacy in the hybrids, supporting the latter scenario. It is possible that the present situation observed in char from Lac Alain arose by repeated backcrossing of female hybrids with male brook char, until the Arctic char nuclear genome eventually disappeared. The lack of nuclear introgression in brook char from Lac Alain further corroborates the hypothesis that complete interspecific replacement of the mitochondrial genome is an ancient event.

The evolutionary impact that the permanent mitochondrial replacement may potentially have on that population is directly related to the neutral or selective nature of variation between the mitochondrial genome of brook char and that of Arctic char. Most if not all intraspecific variation detected in the mitochondrial genome is generally accepted to be neutral. This concept of mitochondrial neutrality has been extended to explain interspecific transfer of mtDNA where introgression has occurred (e.g. Tegelstrom, 1987; Dowling and Hoeh, 1991). It has also been reinforced by theoretical work demonstrating that mitochondrial gene flow across species boundaries is likely, unless the fitness of resulting offsprings is very low (Takahata and Slatkin, 1984). However, the acceptance of neutrality without further consideration of adaptibility has hampered our understanding of the relative roles of deterministic and stochastic processes as driving forces of gene exchange among different taxa (Arnold, 1992). Indeed, a strict test of neutralist versus selectionist hypotheses will require an experimental approach that can substantiate predictions of differential response between introgressed and pure populations (Powers *et al.*, 1991).

The permanent replacement of brook char mtDNA with that of Arctic char could be selectively significant. Differences in the two mitochondrial genomes may be manifested physiologically, since several mitochondrial enzymes, central to intermediate metabolism, are partly encoded by mitochondrial genes. For example, metabolic thermosensitivity in ectotherms has been directly linked to the thermosensitivity of a mitochondrial enzyme, cytochrome oxidase (Blier and Guderley, 1993). The activity of this enzyme, therefore, may reflect the organism's capacity towards thermal adaptation. Genetic differences between the mitochondrial genomes of introgressed and non-introgressed populations could concievably

translate to differences in the metabolic capacity of their mitochondria and, hence, their response to environmental temperature. Indeed, the Arctic char is typically more adapted to cold environments than is brook char, as exemplified by its more northern distribution, and lower thermal optimum (Johnson, 1980; Beamish, 1980). In such a case, we can hypothesize that under low temperature constraints, there is a selective advantage imparted towards introgressed brook char having the mitochondrial genome of Arctic char. This prediction is tested by experimentally assessing differences in thermosensitivity between introgressed and pure brook char individuals at the biochemical (Chapter 4), cellular (Chapter 4), and organismal (Chapter 5) levels of organization.

Clearly, experimentation is needed to determine if the fixation of Arctic char mtDNA in this population has resulted from deterministic (selection) or stochastic (historical) events. Together, experimentation and observation at several levels could potentially provide new insights into selection of both nuclear and mitochondrial gene flow.

2.4 Summary

Although mitochondrial introgression between taxa has been increasingly documented, interspecific replacement of mtDNA is rare, particularly when the donor species is absent. In this chapter evidence is presented for a population of brook char (*Salvelinus fontinalis*) in which all individuals possess the mitochondrial genome of arctic char (*S. alpinus*) despite the present day absence of the latter species in the watershed where the population is located. The mitochondrial genotype of 52 brook char from Lac Alain (Québec) was characterized by RFLP analysis performed over the entire mtDNA molecule

and on a 2.5 kb PCR amplified segment of the ND5\6 region. Although the fish examined were morphologically typical of brook char, the mtDNA of all individuals was identical to the Québec arctic char haplotype. The estimated divergence of this haplotype from previously reported brook char haplotypes is approximately 3%. Together, these results indicate that the mtDNA haplotype observed in Lac Alain brook char has resulted from ancient introgression with arctic char rather than ancestral polymorphism or convergent evolution. They also demonstrate that introgressive hybridization between those two species can have significant and long-term effects on their genetic composition.

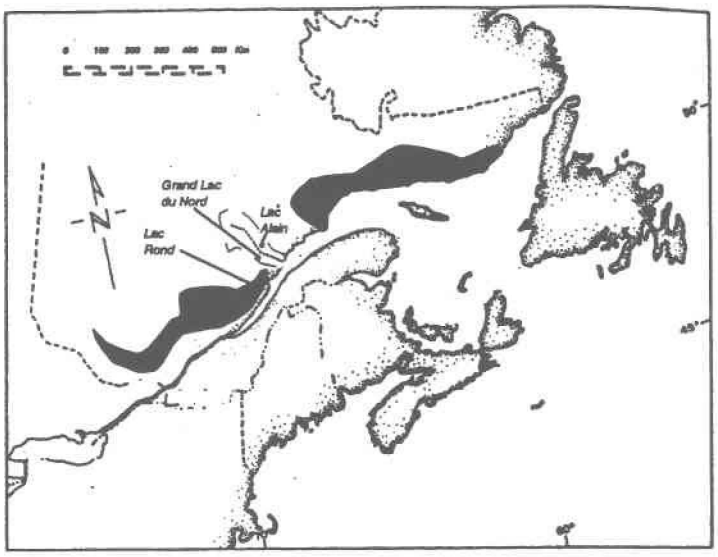

Figure 2.1. Location map of Lake Alain, Lac Rond and Grand Lac du Nord showing present-day distribution of land-locked Arctic char populations on the north shore of the St.-Lawrence River, as well as the limits of the Portneuf River watershed.

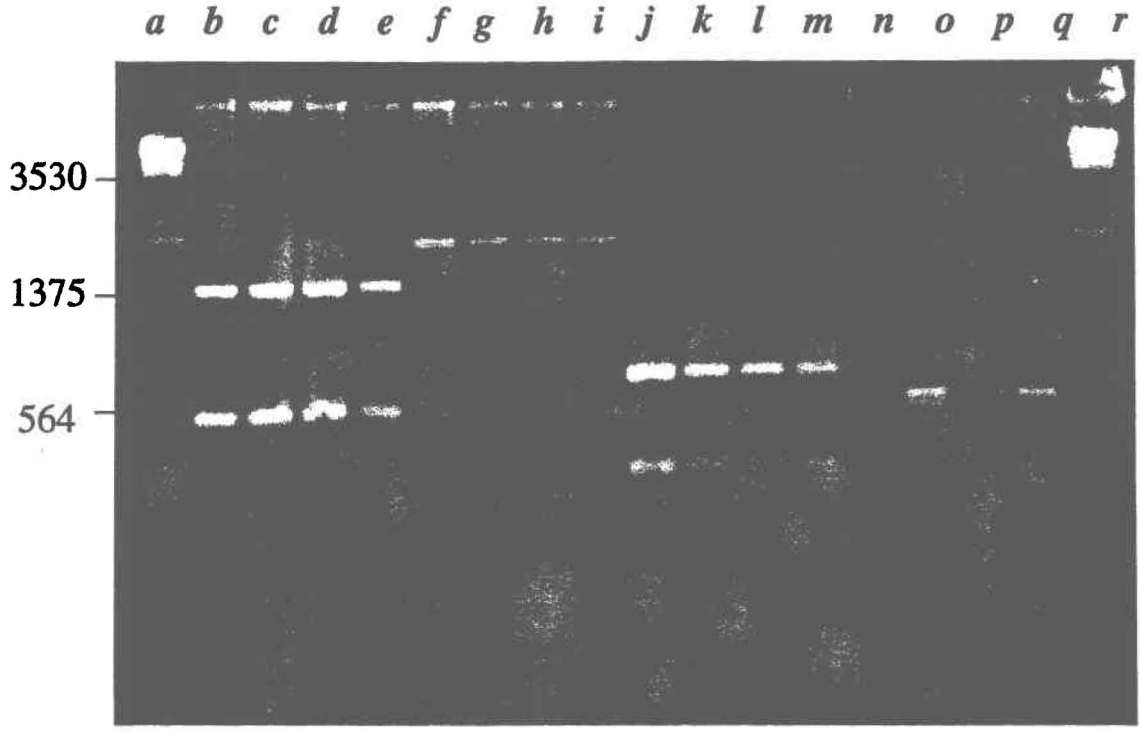

Figure 2.2. Photograph of an ethidium bromide stained 1.2% agarose gel showing restriction fragments produced by digestions of the ND-5/6 amplified segment with *Ava*I (lanes *b* to *i*) and *Hae*III (lanes *j* to *q*). For both enzymes, the first two lanes correspond to individuals from Lake Alain and the next two of Arctic char from Lake Rond (Québec). The four others are of brook trout from Grand Lac du Nord (Québec). Lanes *a* and *r* are lambda phage cut with *Hind*III and *Eco*RI (double digest).

Table 2.1. Allele frequencies at diagnostic loci for brook char, *Salvelinus fontinalis* (SF) and Arctic char, *Salvelinus alpinus* (SA). Introgressed char are from lac Alain and are represented as (LA). The number of fish examined at each locus is given in parentheses.

Locus and allele	**SA (20)**	**LA (30)**	**SF (24)**
IDH-3,4*			
(100/100)	0	1.00	1.00
(130/130)	1.00	0	0
LDH-3*			
($0^a/0^a$)	0	1.00	1.00
(100/100)	1.00	0	0
SDH-1,2*			
(100/100)	0	1.00	1.00
(40/40)	1.00	0	0
SOD*			
(100/100)	0	1.00	1.00
(260/260)	1.00	0	0

[a] The allele present in *S. alpinus* was assigned a value of 100, as the *S. fontinalis* allele remained at the origin.

Table 2.2. List of mtDNA fragments generated by restriction digests of Québec *Salvelinus alpinus*, lake Rond (SA), introgressed lac Alain fish (LA) and Québec *S. fontinalis*, Grand lac du Nord (SF). Presence (1) or absence (0) of different fragment patterns are indicated. Arctic char (SA) samples have fragment patterns typical of Arctic char from southeastern Québec. Brook char samples (SF) are also representative. The number of fish examined is given in parentheses.

Enzyme	**Fragment sizes**						**SA**	**LA**	**SF**
Total mtDNA digests							**(25)**	**(16)**	**(24)**
*Acc*I	6450	5040	2080	1700	1000	500	1	1	0
	8600	3420	2980	1700	480	330	0	0	1
*Bam*HI	15520	1270					1	1	0
	14350	2450					0	0	1
*Bcl*I	8100	7850	1000				1	1	0
	8100	4060	3610	1020			0	0	1
*Bst*EII	9350	4500	2380	580			1	1	0
	7090	4850	4250	560			0	0	1
*Nco*I	7200	6700	1800	1180			1	1	0
	8980	7720	1180				0	0	1
*Nhe*I	7470	7470	1870				1	1	0
	7700	6400	3300				0	0	1
*Pst*I	16800						1	1	0
	16490	310					0	0	1
*Xba*I	13600	3200					1	1	0
	6150	5190	3200	2420			0	0	1
ND- 5\6 segment							**(25)**	**(32)**	**(24)**
*Ava*I	2 000	520					1	1	0
	1450	520	520				0	0	1
*Hae*III	600	600	560	350	350		1	1	0
	730	730	380	380	220		0	0	1
*Hinc*II	1050	900	560				1	1	0
	1050	950	520				0	0	1

CHAPTER THREE

Geographical extent of arctic char (*Salvelinus alpinus*) mtDNA introgression in brook char populations (*S. fontinalis*) from eastern Quebec, Canada

3.1 Introduction

The potential for natural hybridization in freshwater fish is elevated as a result of several factors including external fertilization, unequal abundance of parental species, competition for limited spawning habitat, and susceptibility to secondary contact between recently evolved forms (Hubbs, 1955). There is little information on natural hybridization among species of the salmonid group of fish (Hubbs, 1955; Verspoor and Hammar, 1991). Natural hybridization has been observed between Arctic char (*S. alpinus*) and lake char (*S. namaycush*) in northern Labrador (Hammar et al., 1989) and in the Canadian Arctic (Wilson and Hebert, 1993), as well as between Arctic char and brook char (*S. fontinalis*) in northern Labrador (Hammar et al., 1991). One possible reason for the paucity of reports on natural hybridization between congenerics of *Salvelinus* is the similarity that is sometimes observed in the general appearance of hybrids with one of the parental species (Hammar et al., 1991). This leads one to suppose that natural hybridization between *Salvelinis* species may be much more widespread than currently believed (Verspoor and Hammar, 1991).

A rare consequence of hybridization is introgression which involves an interspecific transfer of either mtDNA or nuclear genes (Verspoor and Hammar, 1991). In the case of mtDNA introgression, interspecific transfers of mtDNA can occur through repeated

backcrossing of female hybrids to males of one of the parental species (Campton, 1987). Repeated backcrossing of female offspring results in hybrid descendants becoming indistinguishable from the parental species based on morphology and nuclear markers, except that they retain the mitochondrial genome of the maternal species.

Introgressive hybridization of foreign mtDNA into a species may eventually lead to complete mtDNA replacement. In such a case, all individuals of an entire population possess the foreign mtDNA. This has been documented for various species in the animal kingdom including grasshoppers, voles, fruit flies, crickets, treefrogs, mice, fish, deer and frogs (see Avise, 1994 for review). In fish, complete mitochondrial DNA fixation is relatively rare having been documented only for minnows occurring in midwestern United States from the *Notropis cornutus/chrysocephalus* species complex (Dowling et al., 1989; Dowling and Hoeh, 1991; Duvernell and Aspinwall, 1995) and recently for *Salvelinus fontinalis/alpinus* (Chapter 2), and for *Salvelinus namaycush/alpinus* (Wilson and Bernatchez, in press) in eastern Quebec. The evolutionary significance of mitochondrial introgressive hybridization is not clear, however it appears that it can have a significant and long-term effect on the genetic composition of the species involved (Chapter 2), and thereby, can contribute to genetic diversity (Dowling and DeMarais, 1993). In fact, Arnold (1992) argues that mtDNA exchange through introgressive events may be more important than mutations as a source of new genetic variability within taxa.

The extent of detected cases of natural introgressive hybridization in fish, and the geographical distribution of introgressed populations are not so well characterized. The extent of mtDNA introgression has only been studied for *N. cornutus* and *N.*

chrysocephalus populations along transects north and south of their hybrid zone in Michigan, Ohio and northern Kentucky (Dowling and Hoeh, 1991) and more recently for populations in Missouri and Arkansas (Duvernell and Aspinwall, 1995). Virtually nothing is known of the extent of mtDNA introgression between *S. namaycush* and *S. alpinus*, nor that of *S. alpinus* and *S. fontinalis* in eastern Quebec.

In North America, the geographical distribution of *S. alpinus* and *S. fontinalis* ranges throughout northern Canada and eastern United States with *S. alpinus* having a more northern distribution and *S. fontinalis* a more southern distribution (Figure 1). Freshwater populations of both species may occur in sympatry where their distributions overlap at the southern extreme of the range of *S. alpinus* and northern end of the range of *S. fontinalis* (Scott and Crossman, 1973). South of 58°N, *S. alpinus* are generally replaced by *S. fontinalis* (Saunders and Power, 1969). In southern Quebec, the known distribution of land-locked *S. alpinus* is limited to less than a hundred lakes. These populations are thought to be vestiges of anadromous populations that lived in the Champlain sea and Atlantic ocean about 12,000 years ago (Power et al., 1973).

In the Côte-Nord region of south-eastern Quebec, a brook char population (*S. fontinalis*) completely introgressed with the mtDNA of Arctic char (*S. alpinus*) was discovered in lac Alain belonging to the Portneuf river drainage (Chapter 2). Introgressed brook char individuals were morphologically indistinguishable from typical brook char and homozygous for diagnostic allozyme alleles and only possessed microsatellite allelles characteristic of brook char (Chapter 2; Chapter 5). Since Arctic char are presently absent

from the Portneuf watershed, it is thought that the original hybridization event between the species probably occurred during the post-glacial recolonization of the area (Chapter 2).

The work presented in this chapter was undertaken in order to examine the extent of mtDNA introgression in brook char populations and to determine the geographical distribution of these populations in drainages of the Côte-Nord region of south-eastern Quebec. Results are discussed in the context of historical and ecophysiological factors that are responsible for shaping the present-day distribution of introgressed populations.

3.2 Materials and Methods

3.2.1 Study Area and Samples

Lac Alain, where the original introgressed brook char population was first discovered, is located in the Rocheuse river, a tributary of the Portneuf river watershed on the north shore of the St-Lawrence river (Figure 3.1, location 17 on map; Table 3.1). Initial sampling efforts were focused on lakes surrounding the Rocheuse river. Populations upstream and downstream of the Portneuf river drainage were also sampled. All populations were sampled either by angling or gill netting. Sampling was extended to populations from all neighbouring major drainages which included the Escoumins, Sault-au-Mouton, Sault-aux-Cochons, Laval, Betsiamites and aux Anglais rivers (Figure 3.2; Table 3.1). A total of 598 fish from 29 sites were thus sampled. All fish collected were identified as brook char (*S. fontinalis*) based on external appearance. Generally, arctic char are not present in these river systems of the Côte-Nord region (Dumont, 1982; M. Braut, Minstère de l'Environnement et de la Faune, Québec, personal communication), except in

the Aux Anglais river drainage where both Arctic and brook char are known to occur sympatrically. In the lake from this latter drainage (lac Sans Baie), 7 arctic char and 10 brook char were collected by gill netting and identified based on external morphological characteristics (M. Braut, Minstère de l'Environnement et de la Faune, Québec). In the field, all specimens were either frozen whole or only the digestive organs were conserved and frozen. The liver tissue was later dissected at the lab and stored at -80 °C until genetic analysis was performed.

3.2.2 Nuclear DNA analysis

Protein electrophoresis was carried out on cellulose acetate using liver tissue homogenates as described by Hebert and Beaton (1989). Four diagnostic enzymes having alleles previously found to be fixed for either brook char or Arctic char were used to distinguish the species' nuclear genomes (Chapter 2). These enzymes included isocitrate dehydrogenase (IDH), 1.1.1.42; lactate dehydrogenase (LDH), 1.1.1.27; sorbitol dehydrogenase (SDH), 1.1.1.14; and superoxide dismutase (SOD), 1.15.1.1. All enzymes examined were resolved by using a tris-glycine buffer system at pH 8.5 and the staining recipes of Herbert and Beaton (1989).

3.2.3 Mitochondrial DNA analysis

Total DNA was extracted from liver tissue using a phenol/chloroform method (Bernatchez et al., 1992). A 2.5 kb portion of the mitochondrial ND5/6 region was amplified by the polymerase chain reaction (PCR) using primers by Cronin et al. (1993).

Amplification conditions are those described in Chapter 2. Amplified DNA was digested with three restriction enzymes (*Ava*I, *Hae*III and *Hinc*II) which generated haplotypes found to be diagnostic between Arctic char and brook char (Chapter 2). Based on fragment patterns generated by the aforementioned restriction enzymes, a composite mtDNA haplotype was designated for Arctic char as (AAA), and for brook char as (BBB). The DNA fragments were separated on 1.2% agarose gels, run for 5 h at 85V, stained with ethidium bromide, and photographed under UV light.

3.3 Results

3.3.1 Allozymes

Based on diagnostic loci having alleles fixed for either brook char or arctic char, all individuals from each population revealed a nuclear genome of brook char (Table 3.2). In no cases were there shared alleles with Arctic char, indicating that introgression by arctic char at these nuclear genes had not occurred in any of the individuals. For the lac Sans Baie populations, where both arctic char and brook char were captured, the allele frequencies were those expected based on *a priori* morphological identification.

3.3.2 mtDNA characterization

Restriction analysis performed on the ND-5/6 segment of mtDNA generated diagnostic haplotypes which were either characteristic of arctic char (AAA) or brook char (BBB) (Table 3.2). MtDNA analysis of populations from the Côte-Nord region of eastern Quebec revealed that introgressed populations were restricted geographically to the

Rocheuse river branch of the Portneuf river drainage basin. All individuals from these seven populations were fixed for Arctic char mtDNA. Coupled with the allozyme analysis of the nuclear genome of these fish, the results show that brook char populations are completely introgressed with the mtDNA of Arctic char, and that hybrids and pure parentals are apparently absent from these lakes. Upstream and downstream of the Portneuf River drainages, populations were found to have the mtDNA haplotype of typical brook char (Figure 3.2; location 13 and 14 on map). Similarly, for neighbouring drainages, all individuals tested from these populations had the brook char mtDNA haplotype.

3.4 Discussion

3.4.1 Historical considerations

A salient finding from the present survey is that introgressed brook char populations are restricted to the Rocheuse river branch of the Portneuf drainage basin in the Côte-Nord region of eastern Quebec, Canada. Elsewhere in the Portneuf drainage and in neighbouring drainage basins, non-introgressed pure brook char populate the lakes while arctic char are completely absent from these drainages. The sequence of events leading to the present-day distribution of introgressed brook char in this region probably required a combination of several elements including: the historical presence of both species, circumstances leading to the break down of reproductive isolating mechanisms, and the necessary biogeographical conditions in combination with possible selection for mtDNA introgression.

Post-glacial recolonization of the Côte-Nord region of eastern Quebec by *S. alpinus* and *S. fontinalis* likely occurred shortly after the retreat of the glaciers approximately

12,000 years ago. It is believed that anadromous arctic char dispersed first during the so-called marine invasion from their Atlantic coast salt water refuge into the Goldthwait sea that then flooded the Côte-Nord region of Quebec (Dumont, 1982). Evidence for the early dispersal of arctic char comes from the presence of land-locked populations at high altitudes in the Côte-Nord region (Dumont 1982) and the cold temperature tolerance of arctic char (Jensen et al., 1989). Since arctic char are presently observed in lakes at altitudes above 600 m it is suggested that invasion likely occurred before the first isostatic movements which would have trapped some anadromous populations at high altitudes (Dumont, 1982). In the Goldthwait sea, summer environmental conditions that prevailed at the time are thought to have been arctic-like with water temperatures approaching 5 °C (de Vernal et al., 1993). Such temperatures are closer to the thermal regime preferred by arctic char (Johnson, 1980) than to that preferred by brook char (Wismer and Christie, 1987).

Continual warming of the climate finally permitted *S. fontinalis* to disperse into eastern Quebec from proglacial lakes formed during the Belleville Fort Anne phase, which they had occupied since their invasion from their Atlantic or Mississipi refuges (Lacasse and Magnan, 1994). Contrary to arctic char, whose present-day distribution is in an area near or within the limits previously delineated by the Goldthwait sea (Dadswell, 1974), brook char were able to disperse more widely throughout the eastern Quebec region (Scott and Crossman, 1973). The extensive distribution in Quebec of brook char is probably owing to their greater ability for counter-current swimming compared to arctic char (Lacasse and Magnan, 1994). Thus, the historical presence of both species together in the

Côte-Nord region of eastern Quebec would have been more limited by the range of recolonization of arctic char than to that of brook char.

Hybridization between *S. alpinus* and *S. fontinalis* likely occurred shortly after the invasion of brook char into the eastern Quebec region 12,000-9800 years ago (Lacasse and Magnan, 1994) where arctic char had already colonized the area. For hybridization to take place, the reproductive isolating mechanisms would have had to be broken down. One factor argued to be responsible for an increase of hybridization in temperate fish is habitat unstability caused by Pleistocene events (Hubbs, 1955). Hubbs (1955) argues that the incidence of natural hybridization between closely-related species was likely to have increased under such conditions, since deglaciation brought about catastrophic changes in climatic conditions which forced dispersal and vastly decreased ecological niche stability. Another factor known to influence the occurrence of natural hybridization is a disparity between the parental population sizes (Hubbs, 1955; Avise et al., 1988). This has been observed in the Fraser River, Newfoundland where natural hybrids between arctic char and brook char occur, but where arctic char numbers largely predominate (Hammar et al., 1991). Presumably, if the proper mates are not at hand, the species that is locally rare is likely to out-cross (Hubbs, 1955).

The distribution of brook char is wide-spread in eastern Quebec (Bernatchez and Giroux, 1991). This observation, coupled with the high genetic diversity for the species observed from allozyme data (McGlade, 1981) compared to that observed in Arctic char (Kornfield et al., 1981; Anderson et al., 1983), suggest historical population sizes of brook char were probably larger than those of arctic char. Thus, the presence of arctic char in

comparatively smaller numbers may have favoured the occurrence of hybridization. Although brook char and arctic char observed sympatrically usually remain genetically distinct, as observed in lac Sans Baie for instance, circumstances leading to the periodic breakdown of reproductive isolating mechanisms do occur, as in the Fraser river (Hammar et al., 1991). For mtDNA introgression to have come about in the Côte-nord region of eastern Quebec, a breakdown of reproductive isolating mechanisms, between *S. alpinus* and *S. fontinalis*, must have occurred followed by repeated backcrossing of hybrids with *S. fontinalis*. A change in habitat or interspecific competition with brook char may have led to the eventual decline and disappearance of arctic char in the watershed.

3.4.2 Ecological and evolutionary considerations

The presence of introgressed brook char in only the Rocheuse branch of the Portneuf river system and nowhere else is intriguing. Arctic char and brook char are known to occur elsewhere in sympatry (Lacasse and Magnan, 1994). One hypothetical explanation for the absence of introgression elsewhere is that the necessary demographic conditions that would have led to this distribution did not occur. This hypothesis cannot, however, be tested. Apparently, introgressed brook char have not dispersed out of the Rocheuse branch. To our knowledge, there are no obvious geographical barriers restricting gene flow from lakes of the Rocheuse branch to the rest of the drainage downstream. Yet, the discrepancy is striking since not a single introgressed fish has been found elsewhere and populations from the Rocheuse branch do not contain a single non-introgressed pure brook char.

An alternative explanation is that introgressed brook char are at a selective advantage in the Rocheuse river watershed. In an experiment specifically designed to investigate the influence of the mitochondrial genome on mitochondrial structure and function, non-equivalence was demonstrated for introgressed and non-introgressed brook char (Chapter 4). Although we were unable to identify a physiological advantage related to swimming metabolism in juvenile introgressed char, it is posssible that such a basis exist during other life-history stages (Chapter 5). In early post-glacial times, such a physiological basis could have been highly advantageous for introgressed fish as the mean water temperature during the growing season was lower than it is presently (Pagé, 1992). Although the selective value of this physiological advantage remains to be firmly tested, it is plausible that through introgressive hybridization, an arctic char mitochondrial genome in a nuclear brook char background was more desirable for survival at cold temperatures. If true, we propose a combination of both historical demographic conditions and selection for mtDNA introgression, rather than pure stochastic processes, as a more plausible mechanism which could have produced the present-day geographical distribution observed for introgressed brook char in eastern Quebec.

There are several examples in the literature illustrating recent or more ancient horizontal mtDNA transfers among vertebrates, e.g. rodents (Ferris et al., 1983; Tegelstrom, 1987), deer (Carr et al., 1986; Carr and Huges, 1993), canids (Lehman et al., 1991), and fishes (Avise and Saunders, 1984; Dowling et al., 1989; Dowling and Hoeh, 1991; Wilson and Bernatchez, in press). Two cases in the literature where the extent of introgression has been studied within and beyond the contact zone of the species are for

pocket gopher (Ruedi et al., 1997) and cyprinid fish (Dowling and Hoeh, 1991; Duvernell and Aspinwall, 1995) species. These systems have some features in common with the brook char system we describe. Firstly, mtDNA introgression is not necessarily limited to the present-day contact zone of the species. In both cases, mtDNA replacement is observed outside the range of contact where the mtDNA donor species is presently absent. Secondly, the species involved have different ecological requirements or opposing geographical distributions which suggest that the species either have different temperature preferenda or did in the past. For example, with pocket gophers, *Thomomys bottae ruidosae* is restricted to high-elevation coniferous forest zones, whereas *T.b. actuosus* occupies lower-elevation woodland areas (Ruedi et al., 1997). Similarily, for the cyprinid species, *Luxilis cornutus* is distributed northerly, while *L. chrysocephalus* has a more southern distribution (Duvernell and Aspinwall., 1995). Interestingly enough, as with the introgressed brook char, in these examples the "colder-adapted" species is the mtDNA donor species. Finally, mtDNA introgression is unidirectional such that reciprocal introgression is generally not observed.

The authors of these studies, and other similar studies, all attribute the phenomenon of introgression to historical shifts in the hybrid zone, coupled with genetic drift since Pleistocene events. While they cannot rule out selection as a force shaping the geographical distribution of these introgressed populations, as population geneticists they argue in a traditional neutralist manner. Since cases are increasingly being uncovered that suggest that the mitochondrial genome may be subject to selective forces (see Ballard and Kreitman, 1995 for review) and may even evolve under thermal constraints (Rand, 1994),

it is becoming important to consider carefully the alternative selectionist hypothesis. This is especially true in light of the experimental results that demonstrate non-equivalence for mitochondrial structure and function between introgressed and non-introgressed brook char (Chapter 4). Thus, the present study not only demonstrates the extent of introgression geographically, but also serves as an example to illustrate that phylogenetic relationships drawn from presumably neutral mtDNA variation could potentially be misleading.

3.5 Summary

The geographical extent of *S. fontinalis* introgressed populations in the Côte-Nord region of eastern Quebec was determined. This survey revealed that introgressed *S. fontinalis* populations are restricted to the Rocheuse river branch of the Portneuf drainage basin. Elsewhere in the Portneuf drainage and in neighbouring drainage basins, non-introgressed pure *S. fontinalis* populations populate the lakes. Arctic char are completely absent from these drainages. These findings suggest that the initial hybridization event between the species is ancient and probably occurred shortly after recolonization of the area by the two species. At that time, the two species would have been in contact and the chances of reproductive isolation mechanisms breaking down would have been high. It is probable that a combination of biogeographical conditions coupled with positive selection for mtDNA introgression led to the present-day distribution of introgressed *S. fontinalis* observed in eastern Quebec.

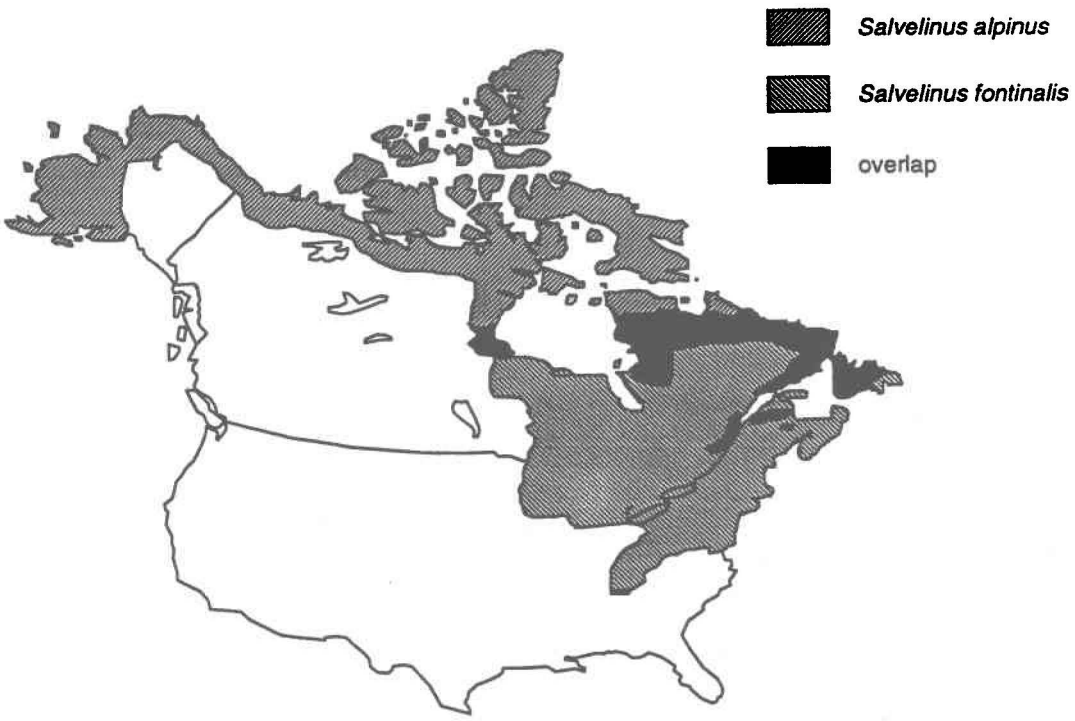

Figure 3.1. Geographical distribution of *Salvelinus alpinus* and *S. fontinalis* and the present overlap zone between the two species in North America (modified from Scott and Crossman, 1973).

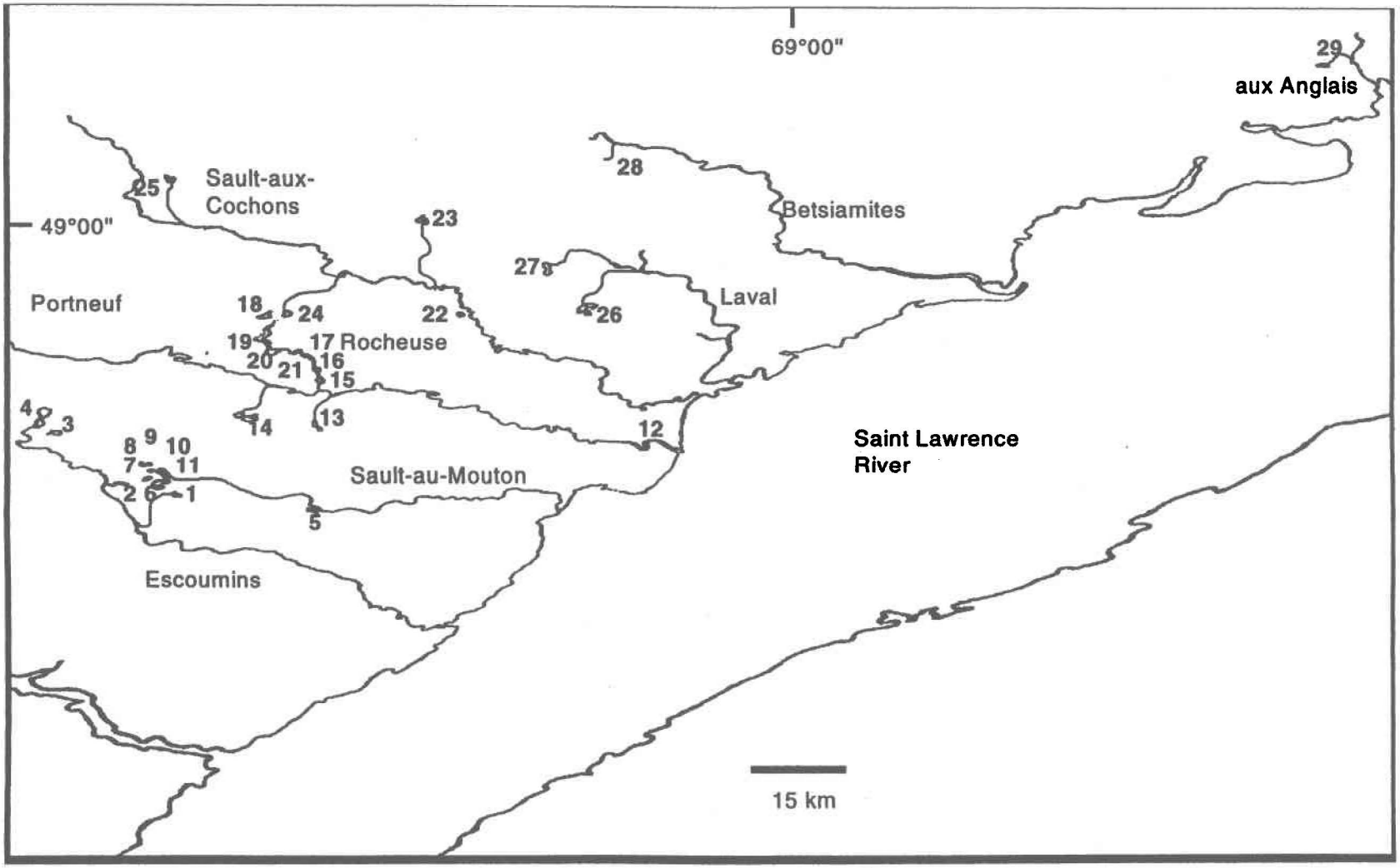

Figure 3.2. Location of watersheds and the sites where brook char populations *Salvelinus fontinalis* were sampled in the Côte-Nord region of south-eastern Quebec.

Table 3.1. Geographical location of populations sampled from various drainage basins in the eastern Quebec region.

Drainage basin and lakes	Location on map	Longitude	Latitude	n
ESCOUMIN				
Cormier	1	48°31′00″	69°43′30″	20
Belanger	2	48°35′00″	69°50′30″	31
Loup	3	48°40′15″	69°56′00″	37
des Coeurs	4	48°42′00″	69°58′00″	36
SAULT-AU-MOUTON				
des Pilliers	5	48°32′15″	69°35′00″	30
des Passes	6	48°33′45″	69°46′15″	7
Brûlé	7	48°35′30″	69°48′15″	24
la Roche	8	48°37′00″	69°48′30″	30
Roger	9	48°36′15″	69°47′45″	4
Féfane	10	48°34′30″	69°47′45″	22
de la Petite Montange	11	48°35′00″	69°47′00″	15
PORTNEUF				
Noir	12	48°43′30″	69°15′45″	12
Boucher	13	48°40′45″	69°35′15″	22
Grand lac du Nord	14	48°41′45″	69°41′00″	26
Rocheuse branch				
Savard	15	48°45′15″	69°35′00″	27
Bourbeau	16	48°46′30″	69°35′15″	12
Alain	17	48°47′45″	69°36′00″	30
Manon	18	48°52′00″	69°39′00″	22
Docile	19	48°51′00″	69°39′15″	12
Micheal	20	48°49′45″	69°39′30″	12
Ruth	21	48°48′00″	69°38′00″	14
SAULT-AUX-COCHONS				
Machoire du Diable	22	48°51′45″	69°23′00″	6
de la Main	23	49°02′15″	69°26′45″	22
Pipe	24	48°51′45″	69°37′45″	7
la Corne	25	49°05′15″	69°47′30″	30
LAVAL				
Croche	26	48°52′15″	69°13′15″	21
Ouellette	27	48°56′45″	69°16′30″	30
BETSIAMITES				
Mille 45	28	49°08′45″	69°14′45″	20
AUX ANGLAIS				
Sans Baie	29	49°17′30″	69°13′30″	17
total				598

Table 3.3. Allele and mtDNA haplotype frequencies for populations from various drainage basins found in the eastern Quebec region.

		Diagnostic loci and alleles				Diagnostic mtDNA haplotype
Drainage basin and lakes	Location on map	IDH-3,4* (100/100) (130/130)	LDH-3* (0/0) (100/100)	SDH-1,2* (100/100) (40/40)	SOD* (100/100) (260/260)	mtDNA (BBB) (AAA)
ESCOUMIN						
Cormier	1	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Belanger	2	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Loup	3	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
des Coeurs	4	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
SAULT-AU-MOUTON						
des Pilliers	5	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
des Passes	6	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Brûlé	7	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
la Roche	8	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Roger	9	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Féfane	10	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
de la Petite Montange	11	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
PORTNEUF						
Noir	12	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0

table 3.3 continued Drainage basin and lakes	Location on map	Diagnostic loci and alleles IDH-3,4* (100/100) (130/130)	LDH-3* (0/0) (100/100)	SDH-1,2* (100/100) (40/40)	SOD* (100/100) (260/260)	Diagnostic mtDNA mtDNA (BBB) (AAA)
Boucher	13	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Grand lac du Nord	14	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Rocheuse branch						
Savard	15	1.0 0	1.0 0	1.0 0	1.0 0	0 1.0
Bourbeau	16	1.0 0	1.0 0	1.0 0	1.0 0	0 1.0
Alain	17	1.0 0	1.0 0	1.0 0	1.0 0	0 1.0
Manon	18	1.0 0	1.0 0	1.0 0	1.0 0	0 1.0
Docile	19	1.0 0	1.0 0	1.0 0	1.0 0	0 1.0
Micheal	20	1.0 0	1.0 0	1.0 0	1.0 0	0 1.0
Ruth	21	1.0 0	1.0 0	1.0 0	1.0 0	0 1.0
SAULT-AUX-COCHONS						
Machoire du Diable	22	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
de la Main	23	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Pipe	24	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
la Corne	25	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0

table 3.3 continued		Diagnostic loci and alleles				Diagnostic mtDNA haplotype
Drainage basin and lakes	Location on map	IDH-3,4* (100/100) (130/130)	LDH-3* (0/0) (100/100)	SDH-1,2* (100/100) (40/40)	SOD* (100/100) (260/260)	mtDNA (BBB) (AAA)
LAVAL						
Croche	26	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Ouellette	27	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
BETSIAMITES						
Mille 45	28	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
AUX ANGLAIS						
Sans Baie (brook char)	29	1.0 0	1.0 0	1.0 0	1.0 0	1.0 0
Sans Baie (arctic char)	29	0 1.0	0 1.0	0 1.0	0 1.0	0 1.0

CHAPTER FOUR

Functional aspects of mitochondrial enzymes and isolated mitochondria in introgressed and non-introgressed brook char populations (*Salvelinus fontinalis*)

4.1 Introduction

The evolutionary significance of genetic variation generated through introgressive events is unclear. It has been suggested that mtDNA variation resulting from introgressive events may be more important than mutations as a source of genetic variation for species (Arnold, 1992). Although there are relatively few reported cases of complete mtDNA fixation in fish, the possibility that a "selective advantage" incurred from the donor species genome has been hinted at in several studies. Still, genetic variation in mtDNA is traditionally accepted to be selectively neutral and consequently population geneticists are generally proponents of the neutralist school.

To study the evolutionary importance of nuclear genetic variation, an experimental approach has been developed and used by a few evolutionary biologists to resolve such neutralist/selectionist controversies. The experimental strategy developed was originally outlined by Clarke (1973; 1975) and involves the biochemical study of allelic isozymes so that predictions of differential responses could be made at various levels of biological organization. As a first step, his strategy requires a detailed biochemical and physiological study of allozymes. Then, based on the nature of the differences found, the function of the enzyme, and the ecology of the organism, a selective factor could be postulated and a

hypothesis generated that establishes a mechanistic link between the selective factor and the gene product (Clarke 1973; 1975). Such an experimental approach could, in theory, be utilized to investigate the evolutionary significance of mitochondrial DNA variation generated by introgressive hybridization.

Mitochondrial DNA (mtDNA) has a gene content that is relatively conserved with 2 ribosomal RNA genes, 22 transfer RNA genes, and 13 protein genes which code for subunits of enzymes functioning in electron transport or ATP synthesis (Anderson et al., 1981; Chomyn et al., 1985; 1986). One important enzyme that functions in electron transport to reduce O_2 is complex IV, known as cytochrome C oxidase (CCO) (Lehninger et al., 1993). CCO makes a substantial contribution to the mass of the inner membrane (15-20% in beef heart mitochondria) (Tzagoloff, 1982). This enzyme has evolved to carry out the four-electron reduction of O_2, as well as to contribute in the maintenance of the proton-motive force across the mitochondrial membrane (Lehninger et al., 1993). The mitochondrial genome codes and synthesizes the 3 largest subunits (of 7) that make up cytochrome C oxidase (Tzagoloff, 1982).

Oxidative phosphorylation is a coupled respiratory process involving the enzymes of electron transport chain and ATP synthetase (Lehninger et al., 1993). This process takes place in mitochondria and is responsible for the generation of ATP which is needed to meet the energetic requirements of organisms. In fish, highly aerobic tissues such as red muscle are necessarily dependent on the performance of mitochondria for the production of ATP. Red muscle in fish functions predominantly under aerobic conditions to power sustained swimming (Rome et al., 1990). Fish mitochondria oxidize a variety of carbohydrate, lipid

and amino acid substrates (Moyes et al., 1990). Since the respiratory enzymes that are partially coded by the mitochondrial genome are important for the production of ATP, the function of red muscle mitochondria in fish may be influenced by a modification of the mitochondrial genome.

A consistent trend in the enzymes of cold-adapted species is that their enzymes have higher catalytic efficiencies than those of warm-adapted species due to the relative flexibilities of the enzymes (Somero, 1995). Hochachka and Somero (1984) suggest that over evolutionary time spans, during which rebuilding of the amino acid sequence of enzymes is possible, adjustments in catalytic efficiency are built into most enzymes. These adjustments are manifested by a change in the number of weak chemical bonds, such as Van der Waals, and hydrogen or ionic bonds, available to stabilize enzyme conformation. Enzymes of cold-adapted ectotherms gain some, or all, of their heightened catalytic efficiency via possession of a more flexible structure, a structure that allows conformational changes to occur with less energy input (Hochachka and Somero, 1984). Q_{10} is a measure of the effect of temperature change on reaction rates and is also referred to as the thermal sensitivity (Hochachka and Somero, 1984). Changes in the thermal sensitivity of an enzyme reaction are a good indication that the structural nature of the enzyme has been altered.

The analysis of Argos et al. (1979) shows that for the large majority of the amino acid substitutions involved in transforming a mesophilic protein into a thermophilic protein, only a single base change in the triplet code is needed. In terms of genetic structure, as well as amino acid composition, the building of thermally efficient enzymes is thus a relatively easy evolutionary task. Nucleotide differences, as observed in mtDNA of closely related species

may be sufficient to influence the catalytic efficiency of the respiratory enzymes they code for and, therefore, be a determining factor for defining the thermal limits of organismal fitness.

A recent discovery of complete fixation of Arctic char (*Salvelinus alpinus*) mtDNA in an allopatric brook char (*S. fontinalis*) population from eastern Quebec (Chapter 2) provides a unique system for investigating the selective importance of mtDNA introgression. In this population, nuclear introgression by arctic char was apparently non-existent based on several diagnostic allozyme (Chapter 2) and microsatellite loci (Chapter 5). In North America, Arctic char and brook char essentially occupy different thermal niches, with Arctic char experiencing considerably colder temperatures than brook char (Scott and Crossman, 1973). In their eastern Quebec range, natural populations of these two species each possess a single mtDNA haplotype (Danzman et al., submitted) with mtDNA sequence divergence between the species estimated at 3% (Grewe et al., 1990).

Using the introgressed brook char described above as a model, an experimental approach similar to what has been used in the past for nuclear genetic variation was applied to investigate the evolutionary significance of mtDNA variation generated through introgressive events. As a first step, the catalytic efficiency, as well as the thermal sensitivity (Q_{10}) of cytochrome C oxidase were determined in red muscle mitochondria from both introgressed and non-introgressed brook char and compared to malate dehydrogenase (encoded entirely by the nuclear genome). In this manner, genetically-determined mitochondrial differences, if any, between introgressed and non-introgressed fish could be evaluated. Given that arctic char mtDNA evolved under more extreme environmental conditions i.e., colder temperatures than brook char mtDNA, the

mitochondrial enzymes of introgress brook char may have heightened catalytic efficiency due to inherent structural differences. These differences may be apparent in the Q_{10} values of enzymes from introgressed and non-introgressed brook char. Such enzymatic properties may consequently be manifested at the sub-cellular level of mitochondrial function allowing the mitochondria of introgressed *S. fontinalis* to function better at cold temperatures. Mitochondrial function was thus, evaluated *in vitro* using oxygen consumption measurements from isolated red muscle mitochondria obtained for several metabolic substrates important in intermediate metabolism.

4.2 Material and methods

4.2.1 Experimental animals

Introgressed brook char (*Salvelinus fontinalis*) were captured by angling in mid-September from lac Alain (48°48′00″, 69°35′30 ″), located in the Portneuf watershed in Quebec. Non-introgressed brook char were obtained from INRS-Oceanology, Rimouski, Quebec and had originated from the Laval river basin, a drainage neighboring the Portneuf watershed. The two fish groups were maintained in similar 350 liter flow-through tanks for 6 months, under a 12:12 light/dark photoperiod, and fed daily on a commercial pellet diet. Water temperature varied according to seasonal temperature and was observed to stabilize at 4.0 ± 1.0 °C by January until time of physiological measurements (February).

4.2.2 Isolation of mitochondria

Fish were killed by a blow to the head and red muscle was rapidly dissected from the lateral line area and placed in 20 ml ice-cold isolation medium (Chamberlin et al., 1991). All subsequent procedures were carried out at 4 °C. The tissue was minced immediately with a straight-edged razor blade and gently homogenized by one pass of a hand-held glass homogenizer, followed by two passes of a mechanically powered Potter-Elvehjem Teflon homogenizer. The homogenate was centrifuged for 10 min at 752 g and the pellet discarded. The supernatant was centrifuged for 5 min at 13,600 g. The pellet was re-suspended in 25 ml of isolation medium and then centrifuged for 5 minutes at 13,600 g. The resulting mitochondrial pellet was re-suspended in isolation medium (1.25 ml) and kept on ice until used for oxidative measurements. An aliquot was also taken for enzyme measurements.

4.2.3 Mitochondrial Enzyme Assays

Enzyme measurements were made on an aliquot of the isolated mitochondrial suspension that had been frozen in liquid N_2. Triton X-100 (final concentration of 0.09%) was added to aliquots of mitochondrial extracts and the suspension was homogenized 2 times for 10 sec bursts at setting 6 with a tissue homogenizer. A subsample of the homogenate was taken for protein determinations prior to the addition of 1 mM glutathione. All preparative procedures were carried out at 4 °C. Enzyme measurements were carried out in duplicate using a Beckman UV-Visible 640 spectrophotometer equipped with a thermostated cell changer. The assays were run at three temperatures, 6, 12 and 18 °C.

Cytochrome-C oxidase (CCO), EC 1. 9. 3. 1: 50 µM reduced cytochrome C (omitted for control); Absorbance change was measured against a control of 50 µM cytochrome C oxidized with 1 mM of $K_3Fe(CN)_6$. Sodium dithionite was used to ensure complete reduction of cytochrome C. Excess dithionite was removed by bubbling the stock cytochrome C solution with air (Hodges and Leonard, 1974).

Malate dehydrogenase (MDH), EC 1. 1. 1. 37: 2 mM NADH, 5 mM oxaloacetate (omitted for control). Reaction rates of MDH were determined by a change in absorbance of NADH at 340 nm (millimolar extinction coefficient ε_{340}, 6.22). Cytochrome oxidase was monitored at 550 nm by following the oxidation of reduced cytochrome C (ε_{550}, 19.1) using a modification of the method used by Blier and Guderley (1988). Total enzyme activities are expressed in units (1 µmol of substrate converted to product per minute) per mg mitochondrial protein. Q_{10} values for enzyme activities were calculated according to Hochacka and Somero (1984).

4.2.4 Mitochondrial oxygen consumption

For oxygen consumption measurements, an aliquot of mitochondrial suspension was diluted 10-fold with reaction medium (Chamberlin et al., 1991) in 1.0 ml water-jacketed respirometers maintained within ± 0.1°C of the incubation temperature (either 4 °C or 12 °C).

The respirometers were sealed and the mitochondrial oxygen consumption was monitored polarographically using a Clark-type electrode. Initial endogenous oxidation rates were estimated in the presence of 0.5 mM ADP and a small amount (0.01 mM) of

malate, which was used to augment the intramitochondrial oxaloacetate pool. Saturating concentrations of substrates were then added to initiate *state 3* oxygen consumption rates as defined by Chance and Williams (1956). Substrates were chosen for their roles in intermediary metabolism and concentrations were those necessary to achieve maximal rates of oxygen consumption in trout (Chamberlin et al., 1991; Blier and Guderley, 1993). For palmitate, oxidation rates were measured by supplementing the reaction medium with 1.0 mM $MgCl_2$, 1.0 mM ATP, 0.6 mM L-carnitine and 0.03 mM CoEnzyme A (Gerrits, 1994). Respiration rates for the mitochondria are presented as nmoles of O_2 consumed per minute per mg of mitochondrial protein. Respiratory control ratios (RCR) were calculated by dividing the *state 3* rate by the *state 4* rate.

4.2.5 Protein

Protein was measured in the mitochondrial suspension and in the isolation medium by the method of Bradford (1976) using bovine serum albumin as the standard. Mitochondrial protein was determined by subtracting the protein concentration of the isolation medium from that of the corresponding mitochondrial suspension.

4.2.6 Chemicals

All chemicals were purchased from Sigma Chemical (St-Louis, Mo).

4.2.7 Statistical Analysis

Statistical differences between groups of brook char were estimated using a Student's t-test (α=0.05) (Steele and Torrie, 1980).

4.3 Results

4.3.1 Mitochondrial Enzyme Activities

Table 4.1 summarizes the mitochondrial enzyme activities at 6, 12 and 18 °C in red muscle mitochondria from non-introgressed and introgressed brook char. At the three temperatures, introgressed char had significantly higher activities for cytochrome C oxidase (CCO) compared to the non-introgressed char. For malate dehydrogenase (MDH), introgressed char had higher activities than non-introgressed char only at 6 °C, while at 12 °C and 18 °C, no differences were observed between groups.

The thermal sensitivities of CCO (Q_{10}) that were calculated for temperature differences of 6 - 12 °C, 12 - 18 °C and 6 - 18 °C, and were all significantly higher in introgressed char compared to non-introgressed char. Conversely, no differences in MDH Q_{10}s were observed between the fish groups.

4.3.2 Mitochondrial Oxygen Consumption

The quality and integrity of the mitochondrial preparations were assessed for each fish. The RCR obtained for malate was used as an index of quality for each mitochondrial preparation; muscle preparations having a RCR of less than 3.5, when malate was the substrate, were excluded from the analysis. The mean increase in oxidation rate resulting

from the addition of 5 mM NADH did not differ significantly between groups of fish (not shown). NADH addition increased malate oxidation relative to the *state 3* rate oxidation by an average of 6.3 ± 0.1% for all fish examined, indicating that membrane permeability of the mitochondria was not compromised by the isolation procedures used.

Tables 4.2 and 4.3 summarize the oxygen consumption rates obtained at 4 °C and 12 °C for isolated red muscle mitochondria from non-introgressed and introgressed brook char. At both temperatures, mitochondria isolated from red muscle oxidized malate and succinate at higher rates than for the other substrates. At 4 °C, the oxidation rates of malate and succinate were significantly higher for introgressed char compared to those of non-introgressed char, while no differences were observed for glutamine, pyruvate and palmitate. At 12 °C, the rates of malate were similar between fish groups, whereas for succinate, non-introgressed char had significantly higher rates compared to introgressed char. Introgressed char had higher rates of pyruvate oxidation compared to the non-introgressed fish, while no differences were observed for glutamine and palmitate between groups.

Relatively higher thermal sensitivities $(Q_{10})^{4\text{-}12°C}$ of malate and glutamine oxidation were observed compared to those of other substrates (Figure 4.2). Q_{10} values for pyruvate oxidation were significantly higher in introgressed fish compared to the non-introgressed fish. No differences were observed between fish groups for the Q_{10} values for the oxidation of the other substrates i.e. malate, succinate, glutamine and palmitate.

4.4 Discussion

4.4.1 Mitochondrial enzyme activity and thermal sensitivity (Q_{10})

Differences in red muscle mitochondrial cytochrome C oxidase (CCO) activity and thermal sensitivity (Q_{10} values) were observed between fish groups, while malate dehydrogenase (MDH) activity only differed 6 °C (Table 4.1). Since differences were mostly observed for CCO, and not MDH, the mitochondrial genome, as such, is likely responsible for this response, given that CCO is partly encoded by mtDNA (Lehninger et al., 1993). Furthermore, a differential response observed in the Q_{10}s of CCO, but not for MDH, is generally consistent with the presumption that the mitochondrial genome could have had an influence on enzyme structure.

Differences in thermal sensitivity (Q_{10}) values for CCO of introgressed and non-introgressed brook char are probably due to inherent structural differences in the enzyme. Generally, cold-adapted species have more flexible enzymes, compared to those of warm-adapted species (Somero, 1995). The basis for these structural differences is in the number of weak bonds used to stabilize protein conformation and these differences are generally reflected in Q_{10} values (Somero and Hochachka, 1984). Apparently, only small nucleotide changes are necessary in the genetic code of thermophillic bacteria to modify the number of bonds used to stabilize enzyme conformation (Argos et al., 1979). Given a sequence divergence of 3 % between arctic char and brook char mtDNA (Grewe et al., 1990), differences in the mitochondrial genetic code of introgressed and non-introgressed brook char may be sufficient to influence the structure of mitochondrial enzyme.

Membrane phospholipids have been shown to activate cytochrome C oxidase (Tzagoloff, 1982). There is some evidence that phospholipids may facilitate the interaction of the enzyme with its substrate, cytochrome C (Tzagoloff, 1982). Although lipid composition was not determined in the present study, the possibility that phospholipid differences exist between introgressed and non-introgressed brook char, and could be responsible for the observed differences in CCO activity, is unlikely. Environmental factors, such as temperature, diet (Hazel and Prosser, 1974; Hazel, 1988) and salinity (Glémet and Ballantyne, 1996; Glémet et al., in press) known to influence membrane lipid composition were kept identical between introgressed and non-introgressed fish. Furthermore, although species-specific differences in mitochondrial membrane composition have been previously reported (Glémet and Ballantyne, 1995), lipid compositional differences in this study were attributable to various osmolytes accumulated by the organisms. Further support for similar membrane lipid composition between *S. fontinalis* groups comes from the results obtained for palmitate oxidation in this study. No difference in palmitate oxidation was observed between *S. fontinalis* populations. Since the transport of palmityl CoA depends on the membrane-bound transporters, carnitine acyltransferases (Hochachka and Somero, 1984), differences in membrane lipids, had they existed, would likely have given rise to differences in the oxidation of palmitate.

4.4.2 Mitochondrial oxygen consumption

The results obtained for oxygen consumption by red muscle mitochondria of introgressed and non-introgressed brook char in the presence of various substrates are less

coherent than those obtained for mitochondrial enzyme activities and thermal sensitivity. This is not surprising since mitochondrial function depends on several integrated components that are not easily teased apart (Lehninger et al., 1993). In addition, the relative importance of each component for the regulation of mitochondrial activity varies according to the respiratory states, i.e., *state 3* or *state 4* (Tager et al., 1983), and are probably in turn influenced by temperature. In contrast to the difficulty in assessing mitochondrial function, measurements of enzyme function are more readily interpretable.

At low temperature, i.e. 4 °C, introgressed char have higher maximal rates for the oxidation of both malate and succinate compared to non-introgressed char indicating that the capacity of mitochondria in introgressed brook char is heightened at cold temperatures for those substrates. These substrates are, however, probably not physiologically important for char red muscle mitochondria (Gerrits, 1994). Nonetheless, the oxidation rates of malate and succinate best approximate the maximal capacity of the electron transport system, and are thus, most susceptible to reflect the adjustments made in the respiratory system. Therefore, our results for malate and succinate oxidation between introgressed and non-introgressed *S. fontinalis* lend support to the hypothesis that functional differences in the electron transport system have resulted from the influence of the mitochondrial genome.

At higher temperatures, i.e. 12 °C, malate oxidation is higher in introgressed *S. fontinalis* compared to non-introgressed fish, however, the reverse is observed for succinate oxidation. The reasons for these observations are not clear. The oxidation of pyruvate is, however, influenced at 12 °C being higher in introgressed char compared to

non-introgressed char. In trout red muscle mitochondria, pyruvate is an important metabolic substrate (Blier and Guderley, 1993). The oxidation of pyruvate may be controlled either by its entry into mitochondria or by its decarboxylation (Patel et al., 1984; Mela-Riker and Bukoski, 1985). Thermal limitations on maximal rates of substrate oxidation could, in theory, be associated with limitations of the electron transport system, as a whole, the adenylate-nuclotide translocator, or even the synthesis of ATP (Lehninger et al., 1993). It is conceivable that enhanced rates demonstrated by introgressed char are attained because of increased efficiency of enzymes functioning in the electron transport system, for example the mitochondrially-encoded cytochrome C oxidase.

The fact that pyruvate is the only substrate where differences in thermal sensitivity (Q_{10}) are observed between fish groups is intriguing. If thermal sensitivity differences are due to an influence from the mitochondrial genome, then one could argue that differences should have been observed for other substrates as well. Halestrap (1975) found that for rat liver mitochondria, the pyruvate carrier is highly thermosensitive (Q_{10}= 4.6). The pyruvate carrier may be responsible for differences in thermal sensitivity observed between introgressed and non-introgressed char.

4.4.3 Evolutionary importance of findings

The biochemical analysis of a protein most likely to be influenced by the mitochondrial genome, i.e., cytochrome C oxidase revealed differences between introgressed and non-introgressed *S. fontinalis* populations. This led to the prediction that at a higher level of biological function, for example the sub-cellular level, the enzymatic

differences would affect mitochondrial function. At the sub-cellular level, the responses were harder to interpret, due to the complexity of regulation of the mitochondrial respiratory system. It remains that differences were observed that could very well be due to the mitochondrial genome. Experiments in the future should be designed to test predictable differences that will confirm an influence of the mitochondrial genome. At this time, although the basis for the differences observed is difficult to interpret, the influence of the mitochondrial genome on the sub-cellular level of mitochondrial function does not seem neutral.

4.5 Summary

Using introgressed *S. fontinalis* as a model, an experimental approach was applied to investigate the evolutionary significance of mtDNA variation generated through introgressive events. As a first step, the activity, as well as the thermal sensitivity (Q_{10}) of cytochrome C oxidase (CCO) were determined in both introgressed and non-introgressed *S. fontinalis* and compared to those of malate dehydrogenase (MDH). Differential responses between the fish groups were mostly observed for CCO, and not MDH, suggesting an influence by the mitochondrial genome, since CCO is partly encoded by mtDNA. Furthermore, the basis for this response is probably structural as indicated by differences in Q_{10} values. This result is consistent with the presumption that the mitochondrial genome, as such, influences enzyme structure, and thus enzyme function. Based on these enzymatic differences, we thus predicted that at the sub-cellular level of mitochondrial function, introgressed *S. fontinalis* would have mitochondria which function better at cold temperatures. Indeed, introgressed *S. fontinalis*

were found to have a heightened mitochondrial capacity based on the oxidation of malate and succinate at low temperatures, compared to non-introgressed *S. fontinalis*. However, at higher temperatures, i.e. 12 °C the results were less clear since for succinate oxidation, the reverse was observed. Still, differences in the rates and Q_{10}s values for pyruvate oxidation were observed at the higher temperatures. These results support the concept of a non-neutral influence of the mitochondrial genome on enzyme structure and function.

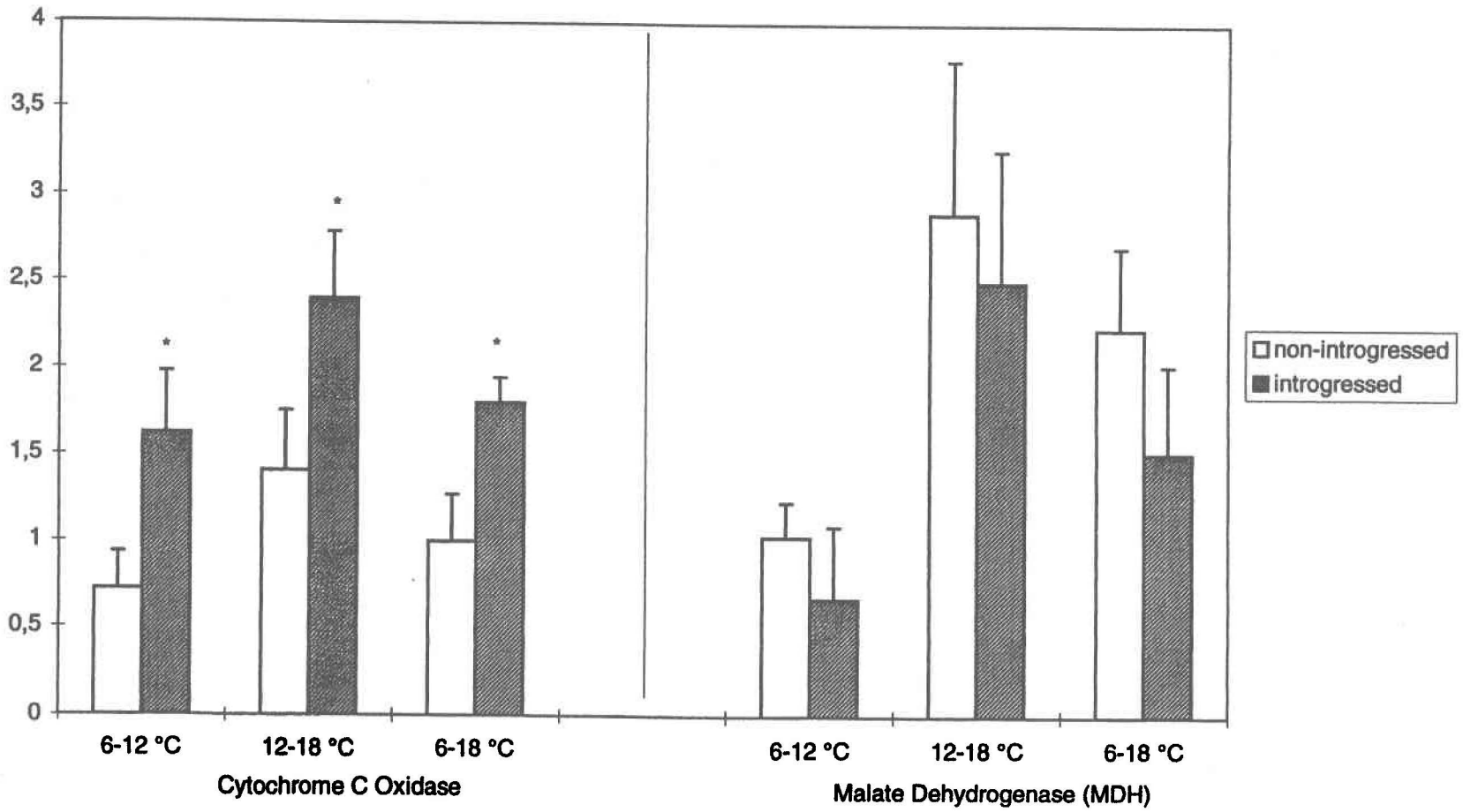

Figure 4.1. Thermal sensitivity (Q_{10}) for cytochrome c oxidase (CCO) and malate dehydrogenase (MDH) in brook char, *Salvelinus fontinalis* populations. All values are presented as means ± s.e. with n=8. Significant differences between groups are indicated by an asterisk (α=0.05).

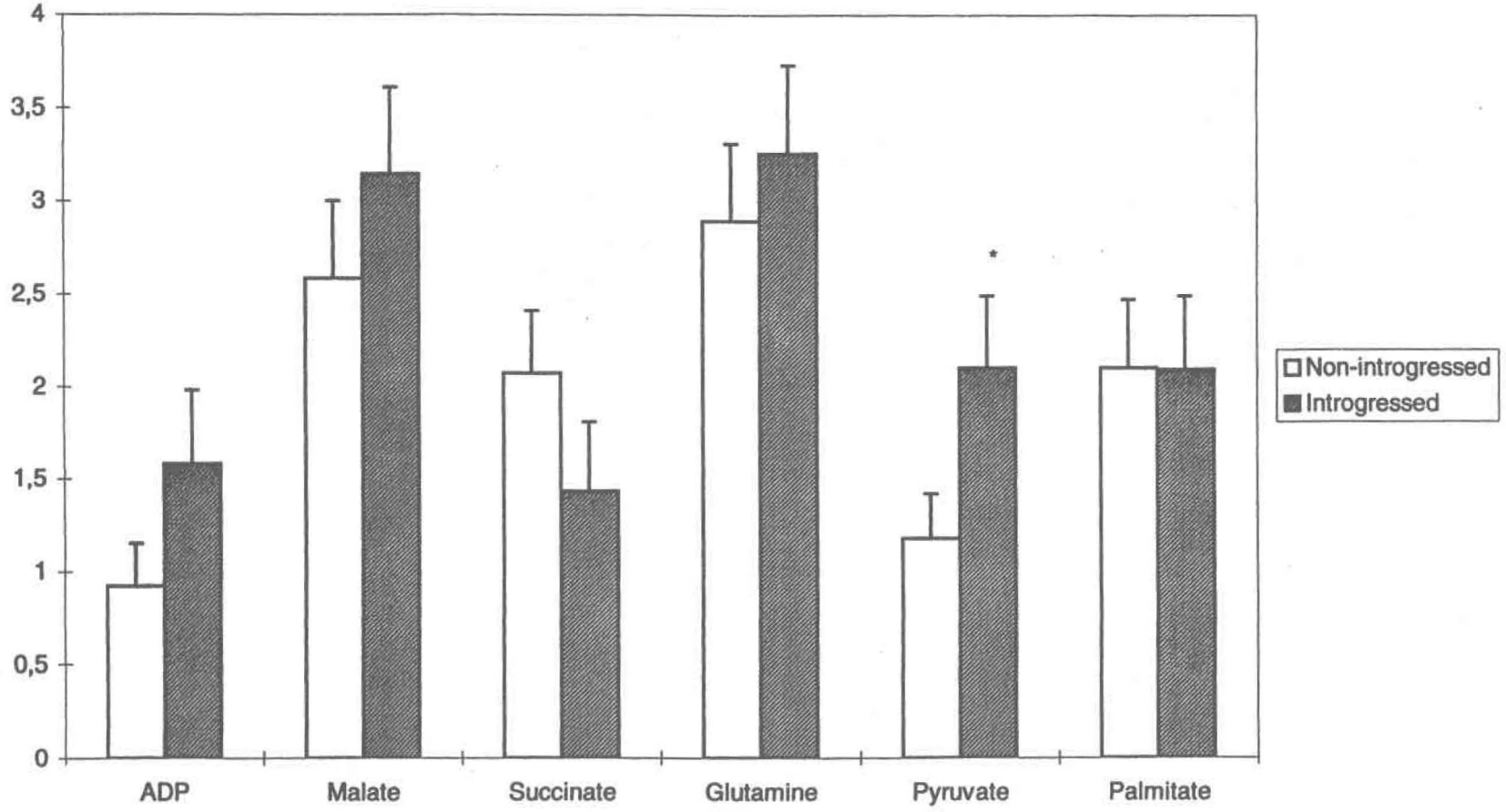

Figure 4.2. Thermal sensitivity $(Q_{10})^{4\text{-}12\,°C}$ for oxygen consumption by red muscle mitochondrial preparations in the presence of several metabolic substrates in brook char, (*Salvelinus fontinalis*) populations. All values are presented as means ± s.e. with n=6. Significant differences between groups are indicated by an asterisk (α=0.05).

Table 4.1. Activities of enzymes in red muscle mitochondria from *Salvelinus fontinalis* populations. Values are presented as means ± s.e. with n = 8. Significant differences between groups are indicated in subscript by different letters (α=0.05). Enzyme activity is expressed in units (µmol of substrate converted to product per min) per mg of mitochondrial protein.

		Non-introgressed	Introgressed
Enzyme	Temp (°C)	µmol· min^{-1}·mg $protein^{-1}$	µmol· min^{-1}·mg $protein^{-1}$
Cytochrome C Oxidase (CCO)	6	$34.1 \pm 4.8_a$	$52.1 \pm 4.6_b$
	12	$38.4 \pm 4.6_a$	$61.8 \pm 5.5_b$
	18	$61.8 \pm 5.8_a$	$96.0 \pm 6.6_b$
Malate Dehydrogenase (MDH)	6	$13.9 \pm 2.0_a$	$21.6 \pm 2.8_b$
	12	20.0 ± 2.2	26.1 ± 2.1
	18	40.7 ± 5.2	49.7 ± 3.9

Table 4.2. Oxygen consumption rates at 4 °C for red muscle mitochondria of non-introgressed and introgressed *Salvelinus fontinalis* populations. RCR - respiratory control ratio. All values are expressed as means ± s.e. with n=6. Significant differences are indicated in subscript by different letters (α= 0.05).

	Non-introgressed		Introgressed	
Substrate	*state3* nmol $O_2 \cdot min^{-1} \cdot$ mg protein^{-1}	RCR	*state3* nmol $O_2 \cdot min^{-1} \cdot$ mg protein^{-1}	RCR
Malate	$17.1 \pm 1.1_a$	4.7 ± 0.5	$20.5 \pm 1.0_b$	4.0 ± 0.3
	3.6 ± 0.3 [1]		5.2 ± 0.6 [1]	
Succinate	$29.6 \pm 1.0_a$	3.2 ± 0.3	$36.6 \pm 1.5_b$	2.7 ± 0.3
	9.7 ± 0.8 [1]		13.9 ± 1.1 [1]	
Glutamine	7.9 ± 0.8		8.2 ± 0.9	
Pyruvate	7.0 ± 0.7		8.0 ± 0.5	
Palmitate	3.5 ± 0.4		4.6 ± 0.6	

[1] *state 4*

Table 4.3. Oxygen consumption rates at 12 °C for red muscle mitochondria of non-introgressed and introgressed *Salvelinus fontinalis* populations. RCR - respiratory control ratio. All values are expressed as mean ± s.e. with n=6. Significant differences are indicated in subscript by different letters (α= 0.05).

	Non-introgressed		Introgressed	
Substrate	*state3* nmol $O_2 \cdot min^{-1} \cdot$ mg protein^{-1}	RCR	*state3* nmol $O_2 \cdot min^{-1} \cdot$ mg protein^{-1}	RCR
Malate	35.9 ± 1.7	4.6 ± 0.6	50.4 ± 1.8	4.3 ± 0.4
	8.9 ± 1.0 [1]		11.6 ± 0.8 [1]	
Succinate	$52.1 \pm 1.6_a$	2.6 ± 0.3	$45.5 \pm 1.7_b$	2.4 ± 0.4
	20.5 ± 1.0 [1]		21.4 ± 1.6 [1]	
Glutamine	18.0 ± 1.3		19.3 ± 1.2	
Pyruvate	$7.5 \pm 0.6_a$		$13.9 \pm 0.8_b$	
Palmitate	6.3 ± 0.7		7.5 ± 0.5	

[1] *state 4*

CHAPTER FIVE

Metabolic scope for aerobic activity and swimming performance in introgressed and non-introgressed brook char populations *Salvelinus fontinalis*

5.1 Introduction

Mitochondrial DNA (mtDNA) polymorphism has been used extensively in evolutionary and population studies for inferring phylogenetic and phylogeographic patterns, population structure, and hybrid zone dynamics (Avise, 1994). Implicit in these studies has been the assumption that mutations and the resulting mtDNA variation are neutral. The concept of mitochondrial neutrality has been advanced to explain mtDNA introgression (Takahata and Slatkin, 1984), a phenomenon resulting from hybridization whereby the mitochondrial genome of one species becomes incorporated into another species through repeated backcrossing (Verspoor and Hammar, 1991). The assumption of mitochondrial neutrality is now being reconsidered as increasing evidence at the molecular level suggests that the mitochondrial genome may be subject to selective forces (reviewed in Ballard and Kreitman, 1995). For instance, selective forces are believed to be partly responsible for maintaining certain mtDNA variants in population cage-type experiments for both invertebrates (MacRae and Anderson, 1988; Fos et al., 1990), and vertebrates (Scribner and Avise, 1994a; 1994b), and in natural fish hybrid populations (Verspoor and Hammar, 1991; Dowling and Hoeh, 1991; Forbes and Allendorf, 1991; Dowling and DeMarais, 1993; Duvernell and Aspinwall, 1995). The possibility that mtDNA variation resulting from introgressive hybridization events could be selectively important for

individual fitness has been suggested, but an experimental basis for this has never been demonstrated.

The functional significance of mtDNA variation is poorly understood. Correlations between mtDNA evolution rates of ectotherms and endotherms and their metabolic rates suggest that the mitochondrial genome evolves under thermal constraints (reviewed in Rand, 1994). The low levels of mtDNA polymorphism found in human Arctic populations have been hypothesized to be maintained through selection for genotypes adapted to extreme climatic and geographical conditions (Malyarchuk and Derenko, 1995). Still, other studies link mtDNA variation more directly to functional oxidative metabolism since mitochondrial genes code for enzyme subunits functioning in electron transport and ATP (Moritz et al., 1987). In fish, highly aerobic tissues such as red muscle depend on mitochondrial oxidative metabolism to meet the ATP demands of sustained swimming (Johnston, 1981). Consequently, energetic parameters obtained from respirometry experiments can be used to assess the "metabolic scope for aerobic activity" (Fry, 1971). The metabolic scope defines the range within which fish function to accommodate aerobic metabolism associated with locomotion and feeding activity (Priede, 1985), and is presumably influenced by the mitochondrial genome.

A recent discovery of complete fixation of Arctic char (*Salvelinus alpinus*) mtDNA, with apparent absence of nuclear gene introgression in an allopatric brook char population (*Salvelinus fontinalis*) (Chapter 2) provides a unique model for investigating the selective importance of mtDNA variation in relation to temperature. In North America, Arctic char and brook char essentially occupy different thermal niches, with Arctic char occurring

mainly in cold arctic waters while brook char experience relatively warmer water temperatures owing to their more southerly distribution and habitat preference (Scott and Crossman, 1973). In their eastern Quebec range, these two species each possess a single mtDNA haplotype (Danzman et al., submitted), with sequence divergence estimated at 0.03 substitution per site (Grewe et al., 1990), a value typical of intraspecific divergence level in most more southerly distributed species (Billington and Hebert, 1991). In order to test the null hypothesis that brook char introgressed with Arctic char mtDNA are not physiologically advantaged over non-introgressed brook char at low temperatures, the aerobic scope for activity and swimming performance were evaluated for populations representing different mtDNA haplotypes under controlled conditions.

5.2 Materials and Methods

5.2.1 Genetic characterization of fish and experimental conditions

Adult introgressed brook char were obtained from the Portneuf river system (Chapter 2) and non-introgressed brook char originated from a neighboring drainage, Laval River (48°50', 69°03') in eastern Quebec, Canada. Genetic characterization of the mitochondrial and nuclear genomes of these two fish groups revealed that the introgressed brook char had the mtDNA of Arctic char in an otherwise brook char nuclear background (Chapter 2). Further analysis of the nuclear genome using microsatellite loci confirmed a brook char nuclear background for introgressed fish (Table 5.1). Introgressed brook char were compared to genetically non-introgressed brook char, so that the main genetic difference between fish groups was their mtDNA haplotypes. F_1 progeny of both fish

groups were reared and maintained under identical laboratory conditions for 18 months. Water temperature in holding tanks was allowed to vary with seasonal temperature, and ranged from 2 °C to 15 °C. Photoperiod was 12 D/ 12L and both fish groups were fed daily *ad libitum* a diet of commercially prepared pellets. Six weeks prior to experimentation, fish were acclimated to 6 °C. Water current in holding tanks was maintained at 15 cm s^{-1} in order to train fish to swim against a current.

5.2.2 Swimming respirometry

Fourteen fish were randomly selected from each group and deprived of food and light for 24 hours, before being placed individually in flow-through modified Blazka-type (3.4 ± 0.05 liter) respirometers (Waiwood and Beamish, 1978). Six identical respirometers were used simultaneously. Fish were allowed to acclimate overnight in respirometers at a low current speed (15 cm s^{-1}). Introgressed (weight 20.54 ± 6.96 g; length 13.15 ± 1.61 cm) and non-introgressed brook char (weight 10.94 ± 3.01 g; length 11.01 ± 0.94 cm) were forced to swim against an increasing current speed, adjusted 5 cm s^{-1} every hour until the fish became exhausted. In order to prevent fish from resting against the back grill of the respirometer, a mild electrical shock was applied. Exhaustion was judged by the failure to respond to the electrical stimulus. Exhausted fish were observed to recover completely upon return to holding tank. Oxygen concentration in each respirometer was measured twice every hour with an interfaced O_2 meter that was calibrated daily using the Winkler method. For comparison purposes, O_2 consumption of swimming fish was measured at three separate temperatures, 6 °C, 12 °C and 18 °C, that

are in the typical range of those experienced by char during the growing season (Jobling, 1981). Fish in holding tanks were acclimated to new temperatures (i.e., 12 and 18 °C) by a gradual thermal increment of 1 °C day^{-1}. Adjustment of water flow through respirometers permitted O_2 concentration to remain above 85% saturation during the experiment. An ott-C2 current meter (Ott hydrometrie; Kempten, Germany) was used to calibrate current velocity in the respirometer.

5.2.3 Calculation of energetic parameters and statistical analysis

Oxygen consumption rate (VO_2) at incremental speeds was expressed as mg O_2 h^{-1}. The swimming speed (current velocity) was corrected for "solid blocking" when cross-sectional area of fish exceeded $1/10^{th}$ that of the respirometer (Bell and Terhune. 1970). Standard metabolic rate (SMR) was determined by extrapolating the log oxygen consumption rate versus swim speed relationship to zero swimming speed (Brett, 1964). Oxygen consumed at maximal sustainable speed (VO_2max) and swimming performance, i.e., critical swimming speed (U_c), was also calculated as described by Brett (1964). A one-way ANOVA with fish weight as a covariant was utilized for all statistical analyses (Steele and Torrie, 1980).

5.3 Results

5.3.1 Metabolic scope for aerobic activity

At 6 °C, introgressed brook char exercised to fatigue displayed a metabolic scope for aerobic activity similar to that of non-introgressed brook char. Similarly, no significant differences were observed at 12 °C or 18 °C (Figure 5.1). No statistical differences in O_2 consumption at maximum sustainable speed (VO_2max) were found between introgressed char and non-introgressed char at the three measured temperatures, 6 °C, 12 °C and 18 °C (Figure 5.1). The standard metabolic rate (SMR) also did not differ between groups ($P>0.05$) at the three measured temperatures, 6 °C, 12 °C and 18 °C (Figure 5.1).

5.3.2 Net cost of swimming

Since an important component of metabolic scope includes the aerobic metabolism associated with locomotory movement, the net cost of swimming was determined for both introgressed and non-introgressed char across a range of swimming speeds. At 6 °C, no statistical difference in the net cost of swimming was observed between introgressed brook char and non-introgressed brook char over the entire swim speed range (Figure 5.2A). Similarly, there were no significant differences in swimming cost at 12 °C and 18 °C over the swim speed range between introgressed and non-introgressed char (Figure 5.2B, 5.2C). Generally, fish swam at progressively higher swim speeds with increasing temperatures.

5.3.3 Metabolic scope associated with feeding metabolism

The proportion of the metabolic scope available for feeding metabolism was calculated by subtracting the net cost of swimming from the total metabolic scope. This analysis revealed that at 6 °C, introgressed char have a similar capacity for accommodating feeding metabolism when compared to non-introgressed char (Figure 5.3A). Similarily, at 12 °C and 18 °C, no significant differences between fish groups were observed over the swim speed range (Figure 5.3B; Figure 5.3C) ($p > 0.05$).

5.3.4. Swimming performance

No significant differences ($p>0.05$) were observed for swimming performance between introgressed and non-introgressed brook char at 6 °C, 12 °C and 18 °C (Figure 5.4). Average critical swimming speeds (U_c) for non-introgressed and introgressed brook char ranged from 20.5 cm s^{-1} to 37.0 cm s^{-1} across temperatures.

5.4 Discussion

5.4.1 Experimental approach

To date, the present study is one of the only few where the influence of the mitochondrial genome was tested at the whole animal level of functioning in fish having different mtDNA haplotypes acquired from introgressive events. This study has used an experimental approach, previously developed to study the evolutionary significance of protein polymorphism (Clarke, 1973; 1975), as a means to discern the importance of mtDNA variation generated through mtDNA introgression. Since the mitochondrial

genome codes for subunits of enzymes functioning in respiratory metabolism (Moritz et al., 1987), it is likely that differences in mtDNA gene products could influence the aerobic capacity of individuals. We have previously demonstrated that differences exist between introgressed and non-introgressed brook char for the catalytic efficiency and thermal sensitivity (Q_{10}) of a mitochondrial enzyme, cytochrome C oxidase, which is partially encoded by mtDNA (Chapter 4). The activity of this enzyme was measured in red muscle mitochondria. Based on this finding, we thus predict a greater metabolic scope for aerobic activity for introgressed brook char at cold temperatures. This aerobic parameter is determined by swimming respirometry during sustained swimming, that is powered by red muscle tissue, and thus relies indirectly on the function of mitochondrial enzymes.

In other studies, fish mtDNA haplotypes have been tested for their effect on the timing of developmental processes by the assessment of meristic counts (Forbes and Allendorf, 1991), however the link between mtDNA gene product and individual function in these studies is ambiguous. The influence that mitochondrial gene products, i.e., the respiratory enzymes, may have on the timing of developmental processes that affect meristic characteristics, is not clear. The lack of differences observed in meristic counts between cutthroat trout mtDNA haplotypes was thus interpreted as supporting the assumption of effective neutrality for mtDNA haplotypes. Our study uses an experimental approach that involves predictions, followed by experimental validation based on genetically-determined differences that may exist in the gene product concerned.

The present study is also unique in that there is a clear adaptive hypothesis why certain mtDNA variants would be favored under certain environmental conditions. Since

the mtDNA of arctic char has evolved under extreme cold conditions, mutational differences existing between arctic char and brook char may reasonably be expected to be reflected on respiratory metabolism of fish so that they perform best at low temperatures. We thus predict that introgressed brook char having the mtDNA of Arctic char would demonstrate superior physiological performance at cold temperatures.

5.4.2 Metabolic scope for aerobic activity

Contrary to prediction, no significant difference was observed in the metabolic scope of introgressed and non-introgressed char. Intraspecific variation of metabolic scope for *S. fontinalis* has been determined and is generally small (P.Boily, unpublished data). Similarly, metabolic scope differences observed between cod (*Gadus morhua*) populations are insubstantial (Nelson et al., 1994). In this study, since mtDNA variants are subject to identical laboratory conditions, it is unlikely that factors known to influence the metabolic scope, such as species-specific differences, stage of development and temperature (Priede, 1985), could have masked any differences, had they existed. Since no difference in metabolic scope was apparent at low temperatures, the data are consistent with the hypothesis that the mitochondrial genome of Arctic char which is present in introgressed char, has little influence on their aerobic metabolism at low temperatures.

5.4.3 Metabolic cost associated with swimming

The cost of swimming for introgressed brook char was similar to non-introgressed brook char over the entire swim speed range. Since energetic efficiency is generally considered to be adaptive and to contribute to fitness (Priede 1985), this finding contradicts a positive selection hypothesis for introgressed char. Moreover, since arctic char are known to have the highest swimming cost among conspecifics of the genus *Salvelinus* (Beamish, 1980), it would not have been surprising to observe a higher swimming cost for introgressed fish who have arctic char mtDNA. However, swimming inefficiency has apparently not been retained in the mitochondrial genome of arctic char and suggests that this particular trait related to the influence of mtDNA is not expressed at the individual level.

5.4.4. Potential for feeding metabolism

Further analysis of the metabolic scope reveals that introgressed char have a capacity for accommodating feeding metabolism similar to that of non-introgressed char, at low swimming speeds. A greater capacity for feeding metabolism, had it existed, is consistent with a hypothesis of a selective advantage for introgressed char. At low swimming speeds the probability of exceeding the limits of the metabolic scope while feeding would be reduced since there would be less chance of exceeding the metabolic scope. Theoretical analysis has demonstrated that selection for this type of efficiency can be particularly powerful contributing to the fitness of long-lived multiple brood animals (Priede, 1985). Apparently, introgressed char demonstrate no advantage related to feeding metabolism over non-introgressed char that could benefit individual fitness.

5.4.5. Swimming performance

In fish, sustained swimming ability is an important fitness component because it is related to habitat utilization and vulnerability to predation and, in some species, migration (Taylor and Foote, 1991). Differences in critical swimming speeds for several species of *Oncorhynchus* and their hybrids have been related to morphological differences, and are thought to contribute to the survival of certain populations (Taylor and Foote, 1991; Hawkins and Quinn, 1996). Swimming endurance differences have also been observed for different LDH-B genotypes of the euryhaline fish, *Fundulus heteroclitus* (L.), that experience opposite thermal regimes (DiMichele and Powers, 1982). These studies would seem to indicate that swimming performance is largely under the influence of the nuclear genome since this genome codes for both morphological characteristics and nuclear enzymes such as LDH. The absence of differences in swimming performance in the present study between introgressed and non-introgressed char suggest that nuclear genes are more important than mtDNA in determining swimming performance

5.5 Summary

In summary, we have failed to identified a physiological basis which could be selectively advantageous for introgressed brook char, enabling them to either avoid exceeding their metabolic scope and/or to deploy more energy towards feeding metabolism at low temperatures. Had such an advantage existed, this would have been important at the time of hybridization approximately 12,000 years ago during post-glacial recolonization by Arctic char and brook char when selection pressures at cold temperatures were particularly strong.

Failure to identify a physiological basis for selection may be due to the life history stage used for the assessment of aerobic performance. A physiological advantage may manifest itself at developmental stages, other than the juvenile stage, in the life-history of introgressed fish. For example, it is possible that fish are physiologically advantaged by their aerobic metabolism at the egg stage, or embryo stage or even as adults. Positive selection of introgressed char over non-introgressed char at any of these stages would have led to the present-day introgressed brook char population, as natural selection would still have operated at the individual level. Positive selection for introgressed char cannot be dismissed until a careful assessment of physiological performance is made at all other stages of development at the individual level. Until more experimental investigations of physiological performance are undertaken in introgressed fish, caution should be exercise when inferring population divergence on a purely neutral basis.

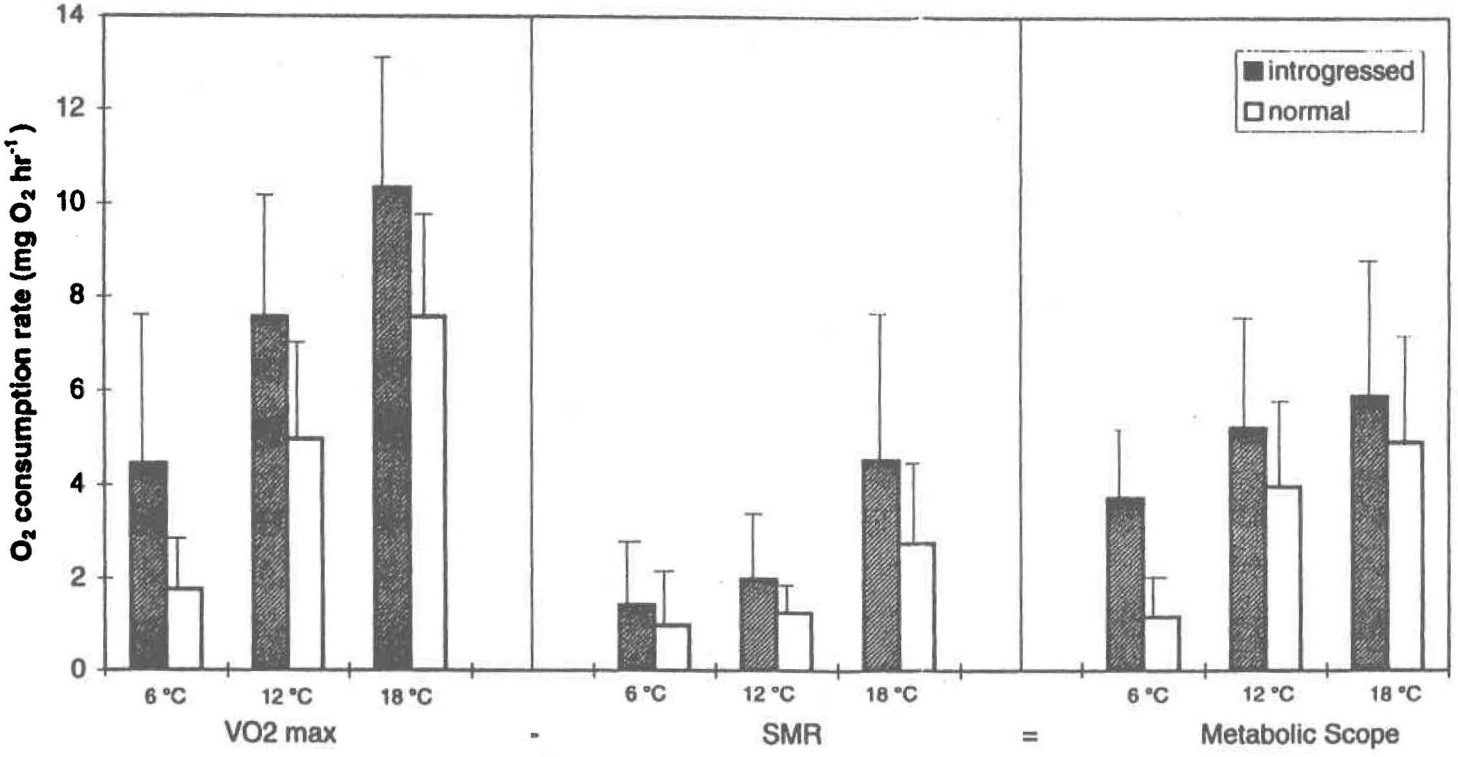

Figure 5.1. Metabolic scope for aerobic activity for introgressed and non-introgressed brook charr, *Salvelinus fontinalis* at 6° C, 12 °C and 18° C. Metabolic scope is expressed as the difference between VO_2 max (maximum metabolic rate) possible during sustained swimming and the SMR (standard metabolic rate). All values are presented as means ± standard deviation ($p > 0.05$).

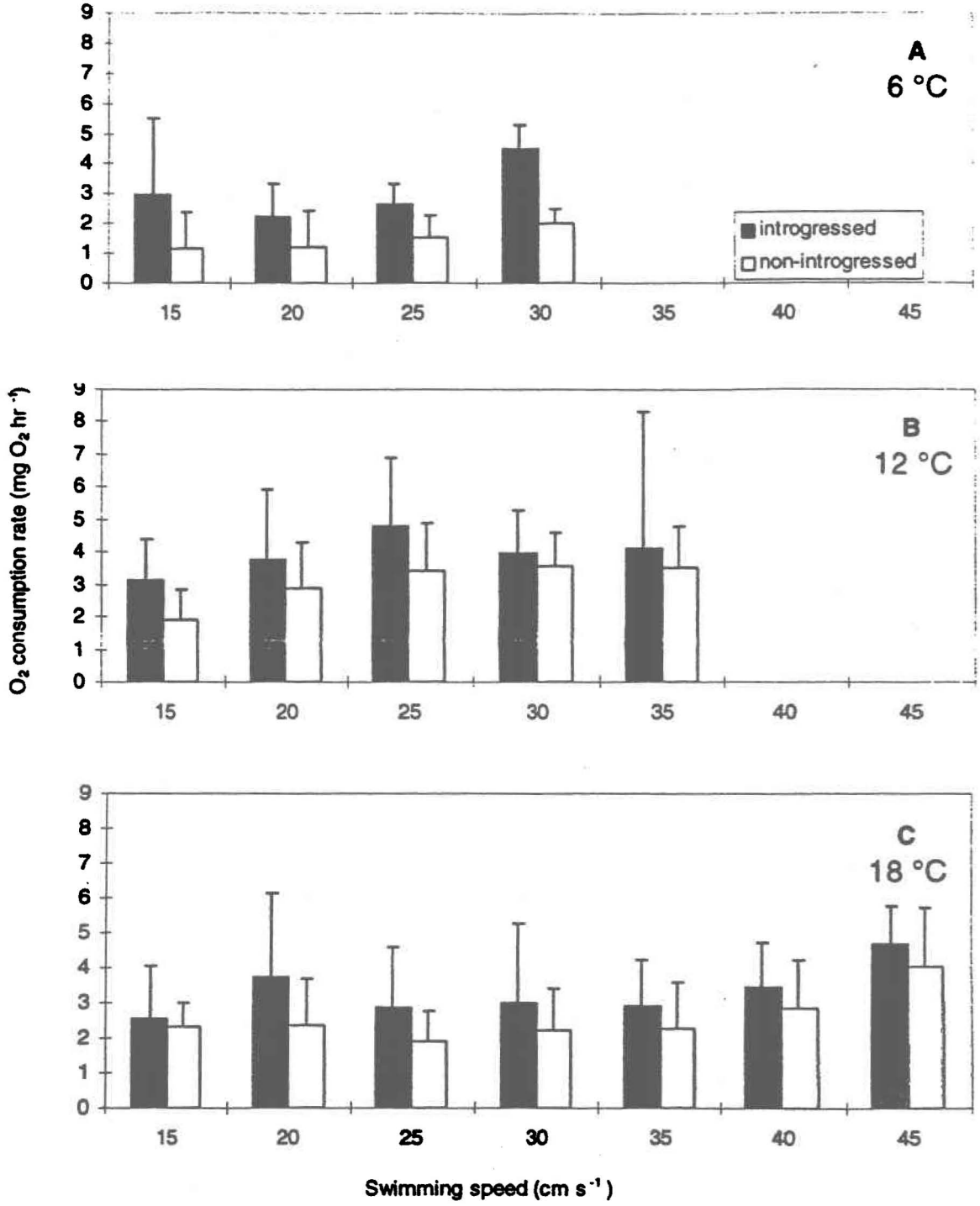

Figure 5.2. Net cost of swimming at a given swimming speed for introgressed and non-introgressed brook charr, *Salvelinus fontinalis* at 6 °C, 12 °C and 18 °C; fig. A, B, and C respectively. Net cost of swimming is expressed as the difference between VO_2 (O_2 consumption rate at a given swimmin speed) and the SMR (standard metabolic rate). All values are presented as means ± standard deviation ($p > 0.05$).

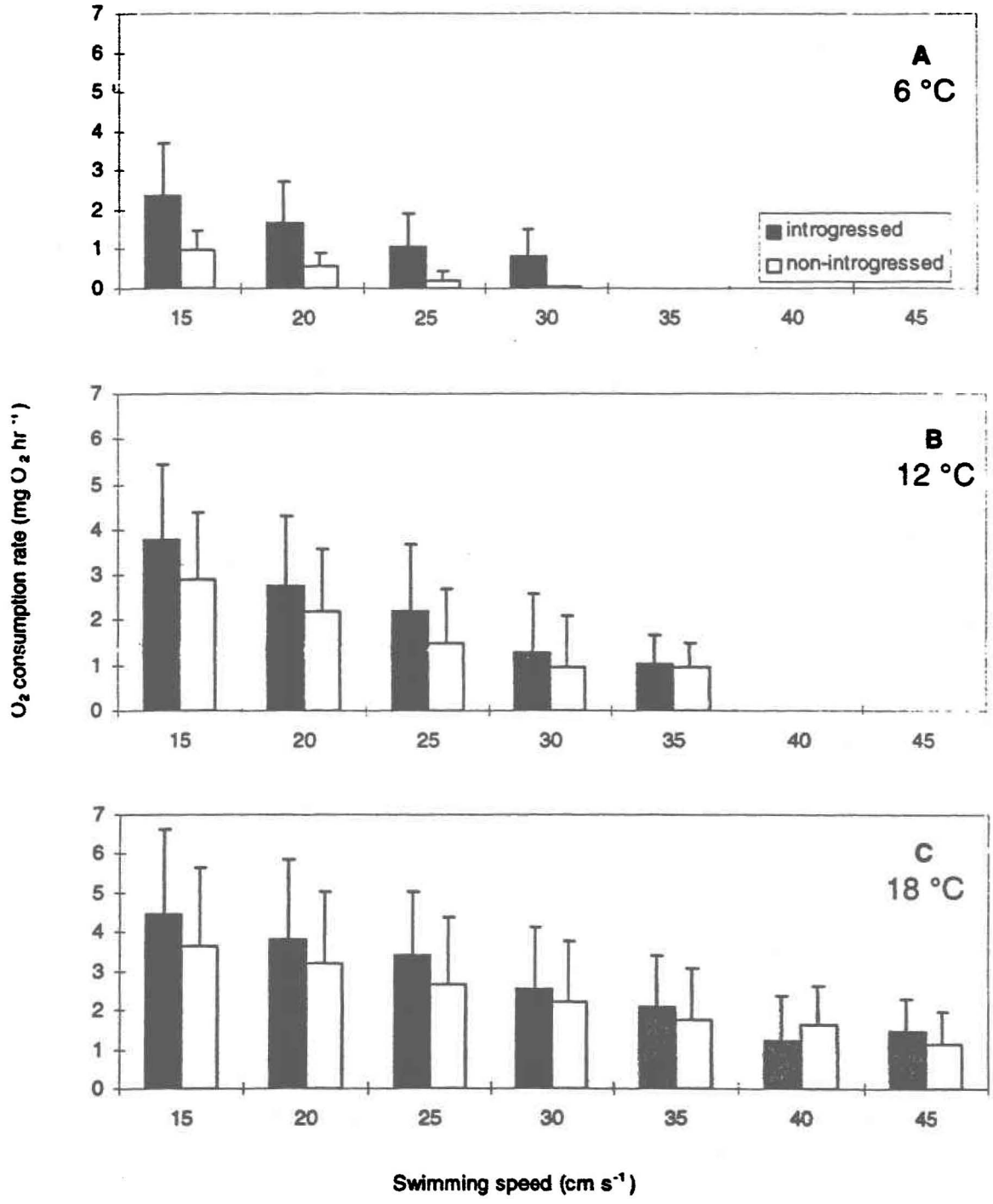

Figure 5.3. Aerobic capacity available for feeding metabolism at a given swimming speed for introgressed and normal brook charr, *Salvelinus fontinalis* at 6 °C, 12 °C and 18 °C; fig. A, B, and C respectively. Aerobic capacity for feeding metabolism is expressed as the difference between the metabolic scope for activity for a given temperature and the cost of swimming at a given speed. All values are presented as means ± standard deviation ($p > 0.05$).

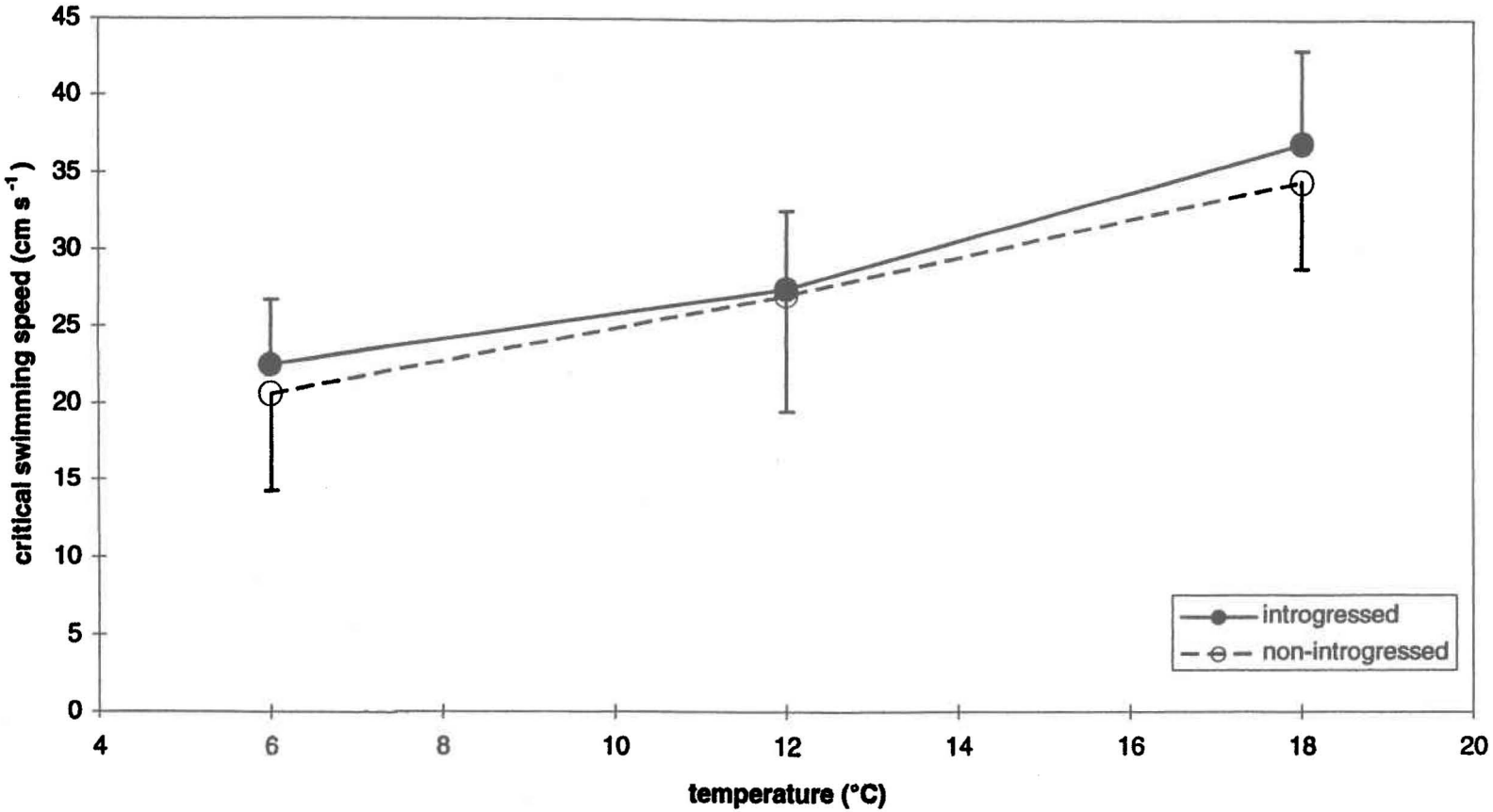

Figure 5.4. Median critical swimming speed in relation to temperature for introgressed and normal brook charr, *Salvelinus fontinalis*. Values are presented as means ± standard deviation. ($p > 0.05$)

Table 5.1. Genetic characterization of the mitochondrial and nuclear genomes of introgressed and non-introgressed brook char using loci/restriction enzymes with alleles/ haplotypes diagnostic for arctic char (AC) and brook char (BC). Values are expressed as allele or mtDNA haplotype frequencies. The number of fish examined for each locus and for mtDNA is given in parentheses for each case.

Nuclear Genome			
allozyme loci[1]	diagnostic allele	introgressed (14)	normal (14)
IDH-3,4		**BC**	**BC**
	(100/100)	1.00	1.00
	(130/130)	0	0
LDH-3		**BC**	**BC**
	(0/0)	1.00	1.00
	(100/100)	0	0
SDH-1,2		**BC**	**BC**
	(100/100)	1.00	1.00
	(40/40)	0	0
SOD		**BC**	**BC**
	(100/100)	1.00	0
	(260/260)	0	1.00
microsatellite loci [2]	diagnostic allele size range		
SFO-11		**BC**	**BC**
	126	1.00	1.00
	122	0	0
SFO-12		**BC**	**BC**
	249-275	1.00	1.00
	225	0	0
SFO-18		**BC**	**BC**
	175-185	1.00	1.00
	161	0	0
Mitochondrial Genome (ND-5,6 segment)			
restriction enzyme[1]	diagnostic mtDNA haplotype	introgressed (14)	normal (14)
***Ava*I; *Hae*III; *Hinc*II**		**AC**	**BC**
	AAA	**1.00**	1.00
	BBB	**0**	0

table 5.1 cont.

	Mitochondrial Genome (ND-5,6 segment)		
restriction enzyme[1]	diagnostic mtDNA haplotype	introgressed (14)	normal (14)
***Ava*I; *Hae*III; *Hinc*II**		**AC**	**BC**
	AAA	1.00	1.00
	BBB	0	0
***Ava*I; *Hae*III; *Hinc*II**		**AC**	**BC**
	AAA	1.00	1.00
	BBB	0	0

[1] methods are described in Chapter 2.
[2] methods are described in Angers et al. (1995) and in Angers and Bernatchez (1996).

CHAPTER SIX

General summary, importance of findings, conclusions and future directions

6.1 General Summary and Importance of Findings

The general objective of this study was to investigate the evolutionary importance of mtDNA introgression, since it is not known whether mtDNA variation, produced through introgressive events, is of neutral consequence or selectively advantageous. An experimental approach, similar to what has been used to study the importance of protein polymorphism, was needed to approach this question. The multidisciplinary approach involved the biochemical characterization of proteins most likely to be influenced by the mitochondrial genome so that predicable differences in sub-cellular physiology, organismal response, etc., could be made. Under experimental conditions, these predictions could then be tested so that the neutralist hypothesis for the influence of mtDNA at that particular level of biological organization could either be accepted or rejected.

A population of brook char (*Salvelinus fontinalis*) discovered in lac Alain, Québec, was first characterized and subsequently used as a model in order to address the evolutionary significance of mtDNA introgression. This population possessed the mitochondrial genome of arctic char (*S. alpinus*) despite the present day absence of this species in the watershed. However, based on allozyme analysis of the nuclear genome, morphological and meristic characteristics, introgressed individuals were identical to genetically pure brook char. These results allowed us to establish that this population was introgressed with the mitochondrial genome of arctic char with no apparent nuclear introgression. In addition, these results

indicated that the mtDNA haplotype observed in lac Alain brook char had resulted from ancient introgression with arctic char rather than ancestral polymorphism or convergent evolution. The results also served to demonstrate that introgressive hybridization between these two species can have significant and long-term effects on their genetic composition.

The geographical extent of introgressed populations of *S. fontinalis* in the Côte-Nord region of eastern Quebec was determined. This survey revealed that introgressed *S. fontinalis* populations are restricted to the Rocheuse river branch of the Portneuf drainage basin. Elsewhere in the Portneuf drainage and in neighbouring basins, pure non-introgressed *S. fontinalis* populations populate the lakes. Arctic char are completely absent from these drainages. These findings indicate that the initial hybridization event between the species was ancient and probably occurred shortly after recolonization of the area by the two species. At that time, the species would have been in contact and the chances of the breakdown of reproductive isolation mechanisms would have been high. It is probable that a combination of biogeographical conditions coupled with positive selection for mtDNA introgression led to the present-day distribution of introgressed *S. fontinalis* observed in eastern Quebec.

As a first step of the experimental approach, enzyme activity, as well as the thermal sensitivity (Q_{10}) of cytochrome C oxidase (CCO) were determined in both introgressed and non-introgressed *S. fontinalis* and compared to the same parameter for malate dehydrogenase (MDH). Differential responses in catalytic efficiency and Q_{10} values between fish groups were observed more for CCO, than for MDH, suggesting an influence by the mitochondrial genome, since CCO is partly encoded by mtDNA. This result is consistent with the presumption that the mitochondrial genome, as such, influences enzyme structure, and thus

enzyme function. Based on these enzymatic differences, the prediction at the sub-cellular level of function was that introgressed *S. fontinalis* would have mitochondria which function better at cold temperatures. Indeed, introgressed *S. fontinalis* were found to have heightened mitochondrial capacity at low temperatures based on the oxidation rates of malate and succinate. Differences in pyruvate oxidation were also observed at higher temperatures, as well as differences in Q_{10} values for pyruvate oxidation which may reflect alterations in CCO structure. Together these results support the premise that the mitochondrial genome influences enzyme structure and function, suggesting that variation in mtDNA may not be neutral.

The aerobic capacity and swimming performance of fish were evaluated using energetic parameters determined by swimming respirometry. Introgressed *S. fontinalis* were found to have a metabolic scope for aerobic activity at low temperature comparable to non-introgressed *S. fontinalis*. Further analysis of the metabolic scope revealed the potential for accommodating feeding metabolism was also similar between the fish groups. These findings indicate that there is no apparent physiological basis related to swimming metabolism identified in this study which could have been of selective value to introgressed char.

6.2 Conclusions and Future Directions

The results from this study demonstrate, for the first time, that mtDNA variation resulting from introgressive events can be functionally important at the molecular and sub-cellular level and may be selectively maintained in natural populations. These findings are

of particular interest to population and evolutionary biologists as mtDNA variation is traditionally believed to be of neutral consequence.

Future research should carry this work further by focusing on several areas including: identification of mtDNA mutations that influence an organism's metabolic capacity, bioenergetic comparisons of mtDNA haplotypes, selection experiments designed to test the hypothesis that certain mtDNA haplotypes are at greater advantage than others at different life-history stages, and finally, thermal constraints influencing mtDNA evolution.

In addition to addressing the basic research issue of physiological adaptation, such research will provide important information about mitochondrial evolution that has not been previously determined. Insights gained by studying the functional and structural properties of gene products will provide fundamental information that will make it possible for population and evolutionary biologists to examine mitochondrial function in the context of phylogenetic relationships. The new knowledge uncovered about the details of temperature adaptation will provide an important scientific basis for a better understanding of the functional significance of mitochondrial genes encoding metabolic enzymes, in general, which may be used to provide insight about other organisms.

A general conclusion of this research is the importance of using a multidisciplinary approach to answer questions regarding the evolutionary significance of genetic variation. It will become increasingly important, in light of the present study, for population and evolutionary biologists to investigate further the evolutionary questions of selection in introgressive systems rather than dismiss mtDNA variation as being of neutral

consequence. Furthermore, the use of molecular techniques in the future may help resolve evolutionary questions that have been so far unapproachable.

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